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The goal of this investigation is to test the hypothesis that estrogen agonists and antagonists promote differential transcriptional activity of the estrogen receptor (ER) by altering accessory protein interactions. We have shown that one or more ER–associated proteins (hsp70, PDI, p48, p45) may be required for maximal interaction of ER with specific DNA sites (EREs) in responsive genes and that the same proteins contribute to the magnitude of DNA distortion without altering the direction of the ER-induced bend of ERE-containing DNA fragments, which is toward the major groove of the DNA helix. We have also used the ligand binding domain of the ER (aa 282-595), expressed in bacteria as a GST-fusion protein (GST-LBD), to capture proteins from mammalian cell extracts that associate with the ER. One protein retained by this technique is a kinase that phosphorylates ER only in the presence of estrogen agonists. Phosphoamino acid analysis and manual sequencing of tryptic peptides have identified the site of phosphorylation as serine 559, a putative casein kinase II phosphorylation site. Ongoing purification of the activity indicates the kinase is a part of a tight-binding complex of at least five proteins, which are ubiquitous and form a stoichiometric complex. Interestingly, this complex, when preassembled on GST-LBD in the presence of estradiol, can be released with a small excess of the estrogen antagonist 4-hydroxytamoxifen, suggesting a mechanism by which the antagonist blocks ER activity. Protein purification is underway to identify the components of this ligand-dependent complex.

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INTRODUCTION

Nuclear receptors represent a family of ligand-inducible transcription factors that control complex events during development, growth, and homeostasis (1). This family includes receptors for the known steroid hormones, thyroid hormone, and vitamins such as retinoic acid and vitamin D, as well as 'orphan' receptors whose ligands remain unknown. Since their discovery in the 1980s, the nuclear receptors have been the object of intense research. Biochemical characterization has defined functional domains required for receptor DNA-binding, dimerization, ligand binding, and transcriptional activation (2). More recent work has uncovered coactivator proteins that interact with several members of the family, adding another layer of complexity to the regulation of receptor activity. Despite these advances, the central question of how nuclear receptor ligands activate transcription at the molecular level remains unanswered.

Work in our lab has focused on understanding the molecular mechanisms that determine estrogen receptor (ER) action. As a member of the nuclear receptor family, the ER mediates estrogenic responses in target tissues including the brain, mammary gland, and tissues of the reproductive tract. Recently, a novel ER cDNA was cloned from rat prostate which was termed ERß (3). The tissue distribution and relative ligand binding of ERα and ERß differ (4) which may be the basis for the selective actions of estrogens in differing tissues. A number of labs are now investigating the role of this newly discovered ER.

The results presented here represent our continued characterization of the process by which ERα (subsequently referred to here as ER) is transformed from an inactive state in the absence of ligand to an activated state. Upon binding estradiol (E2), the receptor undergoes several modifications including dimerization, release and recruitment of associated proteins, and hyperphosphorylation. The carboxyterminal ligand binding domain (LBD) of ER, which has been the focus of our studies, integrates several critical functions including nuclear localization, ligand recognition, dimerization and transactivation. Transcriptional activation is mediated by at least two activation domains in the ER, AF-1 in the N-terminus and AF-2 in the LBD. Evidence suggests that the activity of AF-1 is modulated by growth factors acting through the MAP kinase

pathway (5) while AF-2 activity is responsive to ligand binding (2, 6). Crystal structure analyses of the ligand binding domains of retinoid X receptor α (RXRα) (7), retinoic acid receptor γ (RARγ) (8), and thyroid homone receptor ß (TRß) (9) suggest that a conformational change results from ligand binding, which allows for the realignment of a conserved helical region essential for AF-2 activity. Because point mutations that have no effect on ligand recognition or DNA binding, but abolish transcriptional activity, have been mapped to this putative helix, this region has been predicted to serve as an adapter surface for interactions with other molecules responsible for mediating ER activity.

Transcriptional stimulation by nuclear receptors involves the recruitment or stabilization of the basal transcription machinery in a preinitiation complex (10). The ability of ER to associate with members of the basal transcription machinery, specifically TFIIB (11). and TAF$_{II}$30 (12), has been demonstrated in *in vitro* assays. Importantly, TFIIB and TAF$_{II}$30 were able to stimulate receptor-mediated transcriptional activation. But their interaction with ER was unaffected by mutations in the receptor which abolish transcriptional activity suggesting that the receptor is likely to interact with additional coactivator molecules. Consistent with this theory, several biochemical and genetic approaches have led to the identification of potential coactivator proteins which associate in a ligand dependent manner with members of the steroid receptor family (13). RIP160 (14), RIP140 (15) and p300 (16) were identified by *in vitro* GST-pulldown assays using the ER LBD and were shown to interact directly with the LBD by far western analysis using a [^{32}P]-GST-LBD probe. p140 and p160 are also able to interact with RAR and RXR in a ligand-dependent manner suggesting that these proteins recognize conserved features in the LBD. The yeast two-hybrid and expression screening of bacteriophage cDNA libraries have been used successfully to identify a number of cDNAs that encode interacting proteins including RIP140 (17), Sug1/Trip1 (18), SRC-1/N-CoA1 (19), TIF2/GRIP1 (20, 21), and CBP/p300 (16, 22). SRC-1, TIF2, CBP/p300 markedly stimulate ligand-dependent transcriptional activation by several nuclear receptors and thus can be considered true coactivator proteins. AIB1, a member of the SRC-1 coactivator family, was recently identified in a search

for genes whose expression and copy number were elevated in human breast cancers (23). This altered expression may contribute to development of steroid dependent cancers.

We have been interested in identifying and characterizing novel proteins that selectively associate with the ER-LBD in the presence or absence of estrogen agonists and antagonists and are responsible for distinguishishing between, or effecting, agonistic and antagonistic responses. The differential expression of such coregulators may well play an important role in the observed stimulatory or inhibitory phenotypes of mixed antagonists, like tamoxifen and raloxifene, in the mammary gland, bone, uterus, and vasculature. The relative expression of these co-activators and co-repressors may also play an important role in the effect of mixed antagonists in breast cancers during tumor progression to a more aggressive state. We have used bacterially expressed GST-LBD immobilized on glutathione agarose to capture proteins from mammalian cell extracts. As demonstrated previously, one protein retained by GST-LBD only in the presence of agonists such as estradiol (E2), diethylstilbestrol (DES) and estriol (E3) is a kinase that phosphorylates the ER in a ligand-dependent manner. The partial antagonist 4-hydroxytamoxifen (OHT) and the pure antagonist ICI 182,780 (ICI) were unable to promote phosphorylation by this kinase. Although tyrosine phosphorylation in the ER-LBD has been reported (24, 25), phosphoamino acid analysis revealed that the activity observed by us is a serine/theonine kinase. Serine 167 has previously been identified by amino acid sequencing as a ligand-induced phosphorylation site in human ER when expressed in Sf9 cells (26) and verified in MCF-7 cells. Casein kinase II was able to phosphorylate this site when added to purified receptor in *in vitro* kinase assays. There have been no reports of the consequences this phosphorylation may have on ER activity. More recently, phosphorylation of serine 118 by MAP kinase has been shown to stimulate transcriptional activation through AF-1 in the ER (5). Because the kinase activity we have isolated is specific for the AF-2 region of the receptor and is strongly agonist-specific, it may be important for regulating the transcriptional activity of the ER. Regulation of transcription factor function by phosphorylation can be either positive or negative and has been shown to modulate DNA-binding, transcriptional activation, and/or nuclear transport (27). Our recent work has

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focused on the additional characterization of this interesting kinase. We have mapped the site of phosphorylation to serine 559 and have shown that serine mutants that are unable to undergo phosphorylation are still able to bind the kinase activity. Most interesting are results demonstrating that OHT can cause selective release of the kinase from activated receptor complexes under very mild conditions. These results suggest that OHT may influence ER action by directly influencing the ability of associated proteins to remain bound to the receptor.

MATERIALS AND METHODS

Culture of Mammalian Cells

HeLa, CHO-k1, and CHO-ER cells were cultured in Dulbecco's Modified Eagle Medium/F-12 nutrient mixture (DME/F-12 1:1 mixture, without phenol red, Sigma) supplemented with 10% calf serum (Hyclone), 44 mM $NaHCO_3$, and 1X antibiotics-antimycotic liquid (penicillin, streptomycin, and amphotericin, GibcoBRL). 5 mg/L insulin was also added for CHO-k1 and CHO-ER cells. To maintain the expression and selection of the ER gene, 50 μM $ZnSO_4$ and 40 μM $CdSO_4$ were included in CHO-ER cultures and serum was charcoal stripped to remove steroids. Cells were grown at 37 C in a humidified, 5% CO_2 atmosphere.

For preparation of whole cell extracts, sub confluent cells were released from tissue culture vessels with a non-enzymatic cell dissociation solution (Sigma). The cell suspension was collected, pelleted gently at 1000 rpm for 10 minutes, and washed twice with phosphate buffered saline (PBS). The cell pellet was resuspended in 4 volumes of detergent lysis buffer (50 mM Tris-HCl, pH 7.4, 400 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.25% NP-40) containing protease inhibitors (leupeptin, chymostatin, pepstatin A, antipain, aprotinin and PEFABLOC). Cells were incubated for 20 minutes at 4°C to complete lysis followed by brief sonication to clear lysates. The cell debri was pelleted at 15,000 rpm for 20 minutes and supernatants were frozen in the presence of 5% glycerol and stored at -70°C until use.

Labeling of hER with [^{32}P]orthophosphate

CHO-ER cells were grown to be 80% confluent in complete media. The media was removed and replaced with phosphate-free minimal essential media containing 2% charcoal-stripped and dialyzed serum (2,000 MWCO). After one hour, [^{32}P]orthophosphate (10 mCi/ml, Amersham) was added so that the final concentration was 0.5 mCi/ml media. After 4 hours, cells were stimulated with 10 nM 17ß-estradiol or ethanol vehicle. Cells were harvested after 2 additional hours and lysed using phosphate labeling lysis buffer (20 mM Tris-HCl, pH 7.4, 400 mM NaCl, 2 mM EDTA, 1 mm dithiothreitol, 0.1% NP-40, 10 mM p-nitrophenyl phosphate, 10 mM sodium vanadate, 10 mM sodium pyrophosphate, 10 mM ß-glycerophosphate, and protease inhibitors).

Cell extracts were incubated with a rat monoclonal antibody to ER (H222) for 1 hour at 4 C, followed by immunoprecipitation for 2 hours with rabbit-anti-rat IgG (Zymed) and Protein-A-Sepharose (Pharmacia). Immunoprecipitates were washed 5 times in washing buffer (20 mM sodium phosphate, 400 mM NaCl, 50 mM NaF, 1 mM dithiothreitol, 0.5 mM EDTA, 0.05% NP-40) and boiled in Laemmli sample buffer for 10 minutes. Eluted samples were analyzed by SDS-PAGE and autoradiography.

Production of GST-hER Fusion Proteins

The GST-LBD vector encoding amino acids 282-595 of hER fused to glutathione-S-transferase was transformed into the BL21-pLys strain of *E.coli*. Overnight cultures were diluted 1:10 and grown at room temperature in selective media (LB, 50 μg/ml ampicillin). Cells were induced with isopropyl-ß-D-thiogalactoside (0.1 mM) at an absorbance of 1.5 (at 600 nm). After two hours of induction, bacteria were collected by centrifugation, resuspended in four volumes of detergent lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM DTT, 0.5% NP-40) containing protease inhibitors. Extracts were cleared by sonication followed by centrifugation at 10,000 rpm for 20 minutes. Expression was monitored by western blotting with a rabbit polyclonal antibody against GST and an anti-ER monoclonal antibody (H222). Receptor levels were determined by controlled-pore glass bead (CPG) assay.

Site Directed Mutagenesis

The residue corresponding to serine 559 of hER was mutated to alanine in GST-LBD using the QuikChange^{TM} Site-Directed Mutagenesis Kit (Stratagene) and the following mutagenic primers which had been PAGE purified: 5'-GCC GTG GAG GGG CAG CCG TGG AGG AGA CGG-3' and 5'-CCG TCT CCT CCA CGG CTG CCC CTC CAC GGC-3'. Briefly, plasmid DNA was denatured and oligonucleotide primers containing the desired mutation were annealed. *Pfu* DNA polymerase was used to extend and incorporate the mutagenic primers. Methylated, non mutated parental DNA was digested with *Dpn1* restriction enzyme and the resulting DNA was used to transform XL1-Blue supercompetent cells. Mutations were verified by automated DNA sequencing (University of Chicago Cancer Research Center DNA Sequencing Facility).

GST-Pulldown and *In vitro* Kinase Assays

Bacterial extracts of GST-LBD were preincubated with or without 1 μM of the appropriate ligand for 1 hour at 4 C. Affinity columns were prepared by immobilizing GST-LBD on glutathione-Sepharose-4B (Pharmacia). Columns were washed 5 times with washing buffer containing 20 mM HEPES, pH 7.4, 1.0 M NaCl, 2.5 mM MgCl$_2$, 0.05% NP-40. Mammalian cell extracts were diluted so that the final composition of the buffer was 20 mM Tris-HCl, pH 7.4, 75 mM NaCl, 10 mM MgCl$_2$, 0.5 mM DTT, 20 mM ß-glycerophosphate, and 0.1 mM Na$_3$VO$_4$. Diluted extracts were mixed with prepared affinity columns and incubated for 3 hours at 4 C. Nonspecific proteins were removed by washing with buffer containing 20 mM HEPES, pH 7.4, 400 mM NaCl, 2.5 mM MgCl$_2$, 0.1 mM EDTA, and 0.05% NP-40.

For *in vitro* kinase assays, the pelleted sepharose beads were resuspended in kinase buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 12 mM MgCl$_2$, 2 mM MnCl$_2$, 10 μM Na$_3$VO$_4$, 0.5 mM DTT, 20 mM ß-glycerophosphate) containing 5 μM cold ATP and 1 μCi γ[32P]ATP/5 μl kinase buffer (3000 Ci/mmol, Amersham). After 20 minutes at 30 C, the reaction was terminated by repeated washes in column washing buffer as before. Proteins were eluted by incubation at

95 C in Laemmli sample buffer, resolved by SDS-PAGE, and visualized by Coomassie staining. GST-LBD phosphorylation was analyzed by autoradiography of dried gels.

Tryptic Digestion and Peptide Analysis

Phosphorylated proteins were cleaved on nitrocellulose membranes according to the method of Luo et al. (28). After elution from columns, [^{32}P]-labeled hER or GST-LBD were analyzed by SDS-PAGE and transferred to nitrocellulose. Blots were exposed to film and the bands of interest were excised, Cerenkov counted, and washed 3 times in dH_2O. Membrane pieces were incubated in 0.5% PVP-360/100 mM acetic acid (polyvinylpyrrolidone, M_r 360,000, Sigma) for 30 minutes at 37 C. The membrane was then washed 5 times with dH_2O followed by two washes in 50 mM NH_4HCO_3, pH 8.5. Proteins were digested overnight at 37 C with 5 µg sequencing grade trypsin (Boehringer Mannheim) dissolved in 50 mM NH_4HCO_3, pH 8.5. 300 µl dH_2O was added, samples were spun for 5 minutes in a microcentrifuge, and the supernatant was transferred to a fresh tube. Approximately 80% of the radioactivity was consistently recovered in supernatants. Following lyophylization, peptides were oxidized in 50 µl performic acid, diluted with 1 ml dH_2O, and lyophylized.

Tryptic peptides were separated by RP-HPLC using a Beckman HPLC system. Samples were dissolved in 3 M acetic acid and applied to a C18 column (Microsorb-MV; 4.6 x 250 mm; 100 A; Rainin). Peptides were separated at a flow rate of 1 ml/min with a 3-45% acetonitrile/0.1% TFA gradient over 42 minutes followed by a steep 45-80% acetonitrile/0.1% TFA gradient over 5 minutes. Fractions were collected and Cerenkov counted to determine the elution point of phosphorylated peptides.

For two dimensional tryptic peptide mapping, samples from tryptic digests were dissolved in 10 µl pH 1.9 electrophoresis buffer (formic acid (88%): acetic acid: dH_2O, 25:78:897) and spotted on cellulose coated glass plates (20 x 20 cm, 0.1 mm, Merck). Peptides were separated in the first dimension by electrophoresis in pH 1.9 buffer at 1.5 kV for 30 minutes. Plates were dried for 1 hour and subjected to thin layer chromatography in the second

dimension (n-butanol: pyridine: acetic acid: dH_2O, 375:250:75:300). Plates were dried and exposed to film.

To prepare for manual sequencing or HPLC, phosphorylated peptides of interest were extracted from thin layer cellulose plates (29). After careful scraping of plates, peptides were eluted from cellulose by two sequential washes with 100 μl pH 1.9 buffer, followed by one wash with dH_2O. The efficiency of elution was monitored by Cerenkov counting and repeated elutions were done until a >50% recovery was achieved

Immobilization of Peptides and Manual Sequencing

The method of Sullivan and Wong (30) was used for microsequence analysis of radiolabeled phosphopeptides for the purpose of determining the location of phophorylated amino acids. RP-HPLC fractions which contained radioactivity were pooled, lyophylized, and redissolved in 30% acetonitrile. Samples were spotted on arylamine-Sequelon discs (Millipore) which had previously been placed on a Mylar sheet on top of a heating block set at 50 C. The aqueous solvent was allowed to evaporate over 5 minutes and the disc was removed from the heating block. Covalent linkage was accomplished using the supplied kit reagents. 1 mg of 1-ethyl-3-(dimethylaminepropyl)carbodiimide (EDAC), dissolved in coupling buffer, was added and the disc was incubated for 30 minutes at room temperature. The disc was then washed extensively in water and extracted five times with TFA to remove unbound peptides. Three methanol extractions were performed, followed by cycles of manual Edman degradation.

Each cycle of Edman degradation consisted of the following: (i) Add 0.5 ml of coupling reagent (methanol:water:triethylamine:phenylisothiocyanate (PITC); 7:1:1:1, v/v) and incubate at 50 C for 10 minutes. (ii) Remove the reagent and wash the disc five times with 1 ml methanol. (iii) Dry the disc *in vacuuo* for 5 minutes. (iv) Add 0.5 ml TFA and incubate at 50 C for 6 minutes. (v) Save the TFA wash and extract the disc with 1 ml of a mixture of TFA and 42.5% phosphoric acid (9:1, v/v). (vi) Combine the two washes and measure the amount of radioactivity released and remaining on the disc by Cerenkov counting. (viii) Wash the disc six times with 1 ml methanol.

CK2 Western Blotting

Partially purified cell extracts were separated by SDS-PAGE and transferred to nitrocellulose for western blotting. Membranes were incubated in blocking buffer (3% dry milk/TBS/0.2% Tween-20) for 1 hour at room temperature. Blots were then incubated with 1μg/ml of anti-Human Casein Kinase 2 antibody (Upstate Biotechnology) diluted in a 1% dry milk/TBS/0.2% Tween-20 solution for 1 hour, followed by four 5 minute washes in TBS/0.2% Tween-20. Membranes were incubated in a 1:20,000 dilution of goat anti-rabbit IgG linked to horseradish peroxidase (Santa Cruz Biotechnology) for 1 hour, followed by washing in TBS/0.2% Tween-20. Blots were visualized using the Pierce SuperSignal Chemiluminescent Substrate system

Purification of Kinase Activity

0-30% sucrose gradients were prepared (25 mM Tris-HCl, pH 7.4, 400 mM NaCl, 1 mM EDTA, 2 mM DTT, 1 mM NaN3). Cell extracts were applied to the top of prepared centrifuge tubes and then fractionated for 15 hr at 50,000 rpm in a Beckman L8-70 ultracentrifuge. Gradient fractions were collected sequentially through the bottom of each tube. Fractions were analyzed for kinase activity by GST-LBD *in vitro* kinase assays.

Fractions containing kinase activity were further purified by ion exchange chromatography using the Pharmacia LCC FPLC system equipped with a HiTrapQ (Pharmacia) anion exchange column. Samples were applied in low salt buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, 0.05% NP-40, 0.5 mM DTT) and washed for 10 minutes with a flow rate of 1 ml/min. A linear salt gradient from 50 mM to 1.0 M NaCl was applied for 20 minutes and then the column was washed for 10 minutes in 1.0 M NaCl. Samples containing the kinase activity, as assessed by *in vitro* kinase assays, were pooled.

Fractions were pre-cleared on GST-LBD columns in the absence of estradiol. Supernatants were reapplied to colunns containing GST-LBD in the presence of estradiol. After extensive washing in buffer containing 20 mM Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5 mM MgCl2, 0.05% NP-40, kinase activity was eluted by exchange of OHT for E2 for 30 minutes at room

temperature in exchange buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl$_2$, 0.1 mM EDTA, 1 mM DTT, 0.05% NP-40). Purification was analyzed by SDS-PAGE followed by silver staining.

RESULTS AND DISCUSSION

As part of our ongoing effort to isolate regulatory proteins that interact with ER, we have used the ligand binding domain of human ER, fused to glutathione-S-transferase (GST-LBD), to adsorb proteins from mammalian cell extracts whose association with ER is dependent upon the liganded state of the receptor. This technique was successfully used to identify p140 (RIP140), p160 (SRC-1, TIF2, GRIP1) and p300 as ER-interacting proteins (14-16, 20, 21), We have identified several candidate proteins that recognize the transcriptionally active form of ER. In the course of preliminary studies, *in vitro* kinase assays indicated that at least one of these proteins, which binds to GST-LBD only in the presence of estrogen agonists, is a kinase that can phosphorylate the LBD in a ligand-dependent manner. Phosphoamino acid analysis and manual Edman degradation analysis of tryptic peptides derived from the phosphorylated LBD suggested that serine 559 is the site of phosphorylation. Partial purification by sucrose density centrifugation indicated that the kinase activity represents a protein or a complex of proteins >200 kDa, which are present in both ER-positive and ER-negative cell lines. The results discussed here represent the continued characterization of this phosphorylation as well as our progress toward identification of the kinase involved.

Serine 559 is the site of phosphorylation.

We have previously described the HPLC purification and manual Edman degradation of phosphorylated peptides from GST-LBD. This work suggested that serine 559 of hER was phosphorylated in our *in vitro* assay system. **Figure 1** indicates this site is unique to the human form of the receptor. To verify that this site was indeed identified correctly, site directed mutagenesis was performed. Serine 559 was mutated to alanine in GST-LBD using a PCR-based mutagenesis protocol. DNA sequence analysis verified successful mutagenesis. This mutant

was then expressed in *E.coli* as before and used as a substrate in *in vitro* kinase assays. Partially purified kinase fractions were unable to stimulate phosphorylation of the serine to alanine mutant (**figure 2**, lane 4), confirming the identification of serine 559 as the site of phosphorylation.

Given that most important regulatory phosphorylation sites are evolutionarily conserved in other phosphoproteins, and that serine 559 is not conserved in the ER, it was necessary to investigate phosphorylation patterns of the full length ER in a cellular context. To this end, CHO cells that stably express the full length human ER (31) were metabolically labeled with [^{32}P]orthophosphate in the presence and absence of E2. Cells were starved of phosphate for 1 hour followed by preincubation with [^{32}P]orthophosphate for 4 hours to equilibrate phosphate sources. E2 treatment proceeded for 2 hours and cells were harvested. Analysis of the immunoprecipitated ER shows strong hormone dependent phosphorylation (**figure 3**). As previously discussed, hormone dependent phosphorylation has been reported for the ER. In order to compare the *in situ* and *in vitro* phosphorylation results, phosphorylated ER and GST-LBD were digested with trypsin and peptides were resolved by 2-dimensional phosphotryptic peptide mapping. **Figure 4** demonstrates the results of autoradiography of thin layer cellulose plates. As expected, GST-LBD shows one phosphopeptide. This peptide was re-extracted from the plate and subjected to RP-HPLC analysis and manual Edman degradation. These results matched previous profiles obtained for the phosphorylated GST-LBD (data not shown). Full length hER shows a much more complicated pattern of phosphorylation, which is expected due to the multiple sites of phosphorylation that have been reported for ER. One peptide appeared to coincide with the GST-LBD phosphopeptide and was chosen for further analysis. However, due to low recovery, we were unable to perform manual Edman degradation to locate the position of phosphorylation within the peptide. The RP-HPLC analysis is shown in **figure 5**. Both peptides eluted under similar conditions, although the patterns are not identical. Whether this difference can be accounted for by another posttranslation modification of the peptide derived from CHO-ER cells remains to be determined. We are presently increasing the scale of this experiment with several modifications that promote a more complete tryptic digestion and better recovery of

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peptides from nitrocellulose membranes (28). The modified procedure will allow us to generate enough phosphopeptide for the additional biochemical analyses required to unambiguously identify hormone dependent phosphorylation sites in the full length receptor derived from CHO-ER cells. The recent availability of electrospray tandom mass spectrometry (MS/MS) facilities at the University of Chicago will further aid in the characterization of our tryptic peptides, especially those recovered in trace quantities.

The kinase is recruited to the ER at a site distinct from the phosphorylation site.

To determine which regions of the ER are important for recruitment of the kinase activity, we investigated the ability of short synthetic peptides, which corresponded to regions of the LBD, to inhibit kinase activity. A series of peptides encompassing amino acids 447-565 of hER were tested in *in vitro* kinase/competition assays. In each case, a 100- and 1000-fold molar excess of peptide over LBD was tested. Of the nine peptides tested, only one was able to inhibit kinase activity. This peptide corresponds to amino acids 500-520 of hER (QHQRLAQLLLILSHIRHMSNK). A dose/response analysis indicated that a large excess of peptide was required for inhibition (**figure 6**), most likely due to the low solubility of this peptide at physiological pH. Amino acids 500-520 in hER are highly conserved and predicted to form an amphipathic α–helix. This helix is proposed to be important for receptor dimerization (32, 33) and, interestingly, mutants in this region of the receptor have been shown to be transcriptionally defective (34). To further investigate the mode of inhibition of kinase activity, the competition experiment was repeated with two different incubation conditions. Peptide was preincubated with either the immobilized GST-LBD or with partially purified kinase activity. Unbound peptide was removed by washing the immobilized GST-LBD or by size exclusion chromatography, respectively. If the peptide was inhibiting activity by binding directly to the LBD, possibly affecting the receptor's ability to dimerize, the activity should also be inhibited when the peptide is preincubated with the GST-LBD. What we observed was inhibition only when the peptide was preincubated with the extracts that contained kinase activity (**figure 6,**

lanes 9&10). This result indicates that the peptide may be mimicking the recognition surface for the kinase on the ER and thus it blocks activity by direct competition for this binding site.

We therefore propose that the kinase is recruited to the activated ER through a region that is distinct from the site that is phosphorylated. This model is further supported by experiments with an hER LBD truncation mutant that contains amino acids 297-555 and lacks serine 559. Pulldown and *in vitro* kinase assays showed no phosphorylation of this construct (**figure 7**). Interestingly, when the flowthrough fraction (unretained proteins) from hER(297-555) affinity columns was examined, we observed that the kinase activity was depleted from cell extracts in the presence of E2 (**figure 7**). Furthermore, the depleted kinase activity could be recovered. hER(297-555) affinity columns were first incubated with partially purified kinase and then washed in the presence of high salt to remove nonspecifically bound proteins. 500 mM NaSCN was used to elute retained proteins. After dialysis to remove NaSCN from eluted samples, kinase activity could be reconstituted on GST-LBD columns (**figure 7**). Therefore, although serine 559 is necessary for phosphorylation, amino acids 500-520 appear to be involved in recruitment of the kinase to ligand-activated ER.

The kinase has casein kinase 2-like activity, but is not CK2.

Sequence motif analysis of the serine 559 phosphorylation site revealed that it is a consensus casein kinase 2 (CK2) phosphorylation site. CK2 is a ubiquitous serine/threonine kinase present in the nucleus and cytoplasm of all eukaryotic cells studied to date (35). More than 100 protein substrates for CK2 are known, many of which are involved in cell cycle control and signal transduction, making the enzyme an interesting candidate for study. The Notides lab has reported that CK2 is responsible for hormone dependent phosphorylation of serine 167 in hER. They also demonstrated CK2 phosphorylation of hER *in vitro* (26).

To determine if CK2 was indeed responsible for the hormone dependent phosphorylation of GST-LBD in our studies, we performed western blots with an anti-CK2 polyclonal antibody that recognizes the alpha (44 kDa) and alpha' (40 kDa) subunits of CK2 (Upstate Biotechnology). Fractions representing partially purified kinase activity from HeLa cells were separated by SDS-

PAGE and electroblotted onto nitrocellulose membranes. The results shown in **figure 8** indicate that CK2 is not contained in our purified kinase extracts . Furthermore, we were able to detect CK2 in fractions 7-9 from sucrose density gradients of HeLa cell extracts (**figure 8**). The CK2 protein co-sedimented with an IgG standard (~150 kDa) used as a molecular weight marker in our sucrose density gradient system, consistent with the molecular weight of the active CK2 heterotetramer (130 kDa). Therefore, the kinase responsible for agonist-dependent phosphorylation of GST-LBD is not CK2.

The previously published studies which suggest that CK2 is responsible for the strong ligand-dependent phosphorylation seen for hER, are mostly correlative (36). The fact that serine 559 (SVEE) and serine 167 (STND) have similar consensus sequences raises the interesting possibility that the kinase activity we have followed is in fact responsible for the phosphorylation of serine 167, which is reported to be the major site of hormone-dependent phosphorylation in hER. It follows that the kinase would be recruited to hER as a consequence of the conformational change induced in the ligand binding domain by E2. Once bound, the kinase could then phosphorylate serine 167. Because our fusion protein lacks this site, the kinase is able to phosphorylate serine 559 strongly. We are currently testing this hypothesis using full length hER and our purified kinase extracts in *in vitro* kinase assays.

4-hydroxytamoxifen causes the release of the kinase activity.

The triphenylethylene antiestrogen 4-hydroxytamoxifen (OHT) is a leading therapeutic agent in the treatment of estrogen-dependent primary breast tumors. Chimeric receptor assays indicate that OHT potency is established by inhibition of AF-2 activity in hER. OHT does not appear to inhibit ER binding to estrogen response elements (EREs) (32). These studies suggest that the inhibitory effects of OHT may be a result of the inability of the antagonist to elicit a necessary structural change in the ER, thereby rendering AF-2 inactive. Consistent with this model, several known coactivators of ER action (p140, p160) are unable to associate with the OHT-bound receptor *in vitro* (14, 15).

We have demonstrated that the Ser 559 kinase activity is unable to associate with GST-LBD in the presence of OHT (data not shown). We were therefore interested in determining if OHT may be able to cause the release of the kinase from preassembled GST-LBD complexes. Affinity columns were prepared and partially purified kinase activity from HeLa whole cell extracts was incubated with immobilized GST-LBD in the presence of E2. Nonspecifically bound proteins were removed by washing with high salt. Columns were equilibrated at room temperature to facilitate ligand exchange and incubated with increasing amounts of OHT under mild conditions. After 30 minutes, eluted samples were collected. Columns were washed and re-equilibrated in kinase buffer. *In vitro* kinase assays were used to analyze kinase activity which remained bound to GST-LBD. Autoradiography of the SDS gel indicates that incubation with increasing amounts of OHT reduced the retained kinase activity (**figure 9**). Next, we analyzed the eluted samples for kinase activity after charcoal treatment to remove OHT. These samples, when reapplied to GST-LBD in the presence of E2, were able to phosphorylate the receptor, (**figure 9**) verifying that OHT was able to promote the release of the kinase from ER by ligand exchange. To our knowledge, this is the first demonstration of the specific release of a receptor-associated protein by an antagonist. Therefore, the identification of the kinase becomes even more significant since it may allow us to better understand the molecular mechanisms involved in antagonist action.

The kinase is part of a stoichiometric complex of 5 proteins.

The ability of OHT to release the kinase gives us a tool for specific elution of the protein from affinity columns. This, along with the successful use of sucrose density gradients and ion exchange chromatography, has allowed purification of the kinase activity (schematic, **figure 10**). Briefly, HeLa whole cell extracts were fractionated on a 10-30% sucrose density gradient by ultracentrifugation for 15 hours. Fractions were assayed for activity by *in vitro* kinase assays and those containing activity were pooled. The activity consistently eluted in fractions #2-4, representing a large molecular weight protein or complex of proteins. The salt concentration was adjusted to 50 mM NaCl and samples were applied to an anion exchange FPLC column

(monoQ). Proteins were eluted with a linear salt gradient of 50 mM to 1.0 M NaCl. Fractions were again assayed for kinase activity and samples containing activity were applied to GST-LBD affinity columns in the absence of E2. This step allowed preclearance of ER-associated proteins that are ligand-independent. The flowthrough was applied to a GST-LBD affinity column in the presence of E2 to adsorb the kinase activity. Columns were washed stringently in buffer containing up to 250 mM NaSCN or 1.5 M NaCl without loss of kinase activity (data not shown). The specific release of the kinase activity was achieved using a 10-fold molar excess of OHT (versus GST-LBD-E2).

Eluted proteins were analyzed by SDS-PAGE followed by silver staining. **Figure 11** illustrates the elution profiles from affinity columns. Lane 6 shows five proteins (arrows) that bound to GST-LBD only in the presence of E2, were released with OHT, and appear to be stoichiometric. Some GST-LBD is also found in the eluted material (asterisks) and is presumed to be the result of kinetic exchange during the OHT elution at room temperature.

Current research is focused on the identification of the five associated proteins shown in **Figure 11**. We propose that these proteins form a complex that is able to recognize only the agonist-activated ER. One protein contained in this complex is a kinase capable of phosphorylating the ER. It is likely the kinase is recruited to the ER through another member of the protein complex since binding of the kinase does not require its phosphorylation recognition sequence. We have also demonstrated that amino acids 500-520 are important for this recruitment. Whether more than one protein in the complex is making direct contact with the LBD is unknown. Crosslinking studies are planned to address which protein or proteins are nucleating the complex on ER. Far western analysis of affinity purified, separated proteins has failed, suggesting that the intact complex is required for receptor recognition.

The agonist-specific, ER-associated proteins observed in **figure 11** do not appear to represent any of the known receptor-associated proteins previously described in the literature. The ability of these proteins to recognize only the activated receptor makes them candidate

regulatory proteins. We are currently undertaking a large scale purification from HeLa cells in order to obtain proteins levels necessary for microsequence analysis.

REFERENCES

1. Beato, M., Herrlich, P., and Shultz, G. Steroid hormone receptors: many actors in search of a plot, Cell. *83:* 851-857, 1995.

2. Kumar, V., Green, S., Stack, G., Berry, M., Jin, J.-R., and Chambon, P. Functional Domains of the Human Estrogen Receptor, Cell. *51:* 941-951, 1987.

3. Kuiper, G. G. J. M., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J. A. Cloning of a novel estrogen receptor expressed in rat prostate and ovary, Proc. Natl. Acad. Sci. USA. *93:* 5925-5930, 1996.

4. Kuiper, G. G. J. M., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J.-A. Comparison of the Ligand Binding Specificity and Transcript Tissue Distribution of Estrogen Receptors a and ß, Endocrinology. *138:* 863-870, 1997.

5. Kato, S., Endoh, K., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Mishida, E., Kawashima, H., Metzger, D., and Chambon, P. Activation of the Estrogen Receptor Through Phosphorylation by Mitogen-Activated Protein Kinase, Science. *270:* 1491-1494, 1995.

6. Danielian, P. S., White, R., Lees, J. A., and Parker, M. G. Identification of a conserved region required for hormone-dependent transcriptional activation by steroid hormone receptors, EMBO J. *11:* 1025-1033, 1992.

7. Renaud, J.-P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., and Moras, D. Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha, Nature. *375:* 377-382, 1995.

8. Renaud, J.-P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., and Moras, D. Crystal structure of the RAR-gamma ligand-binding domain bound to all-trans retinoic acid, Nature. *378:* 681-689, 1995.

9.   Wagner, R. L., Apriletti, J. W., McGrath, M. E., West, B. L., Baxter, J. D., and Fletterick, R. J. A structural role for hormone in the thyroid hormone receptor, Nature. *378:* 690-697, 1995.

10.   Goodrich, J. A., Cutler, G., and Tjian, R. Contacts in context: promoter specificity and macromolecular interactions in transcription, Cell. *84:* 825-835, 1996.

11.   Ing, N., Beekman, J., Tsai, S., Tsai, M.-J., and O'Malley, B. Members of the steroid receptor superfamily interact with TFIIB (S300-II), J. Biol. Chem. *267:* 17617-17623, 1992.

12.   Verrier, C. S., Roodi, N., Yee, C. J., Bailey, L. R., and Jensen, R. A. High-Mobility Group (HMG) Protein HMG-1 and TATA-Binding Protein-Associated Factor $TAF_{II}30$ Affect Estrogen Receptor-Mediated Transcriptional Activation, Molecular Endocrinology. *11:* 1009-1019, 1997.

13.   Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S., and Tung, L. Nuclear Receptor Coactivators and Corepressors, Molecular Endocrinology. *10:* 1167-1177, 1996.

14.   Halachmi, S., Marden, E., Martin, G., MacKay, H., Abbondanza, C., and Brown, M. Estrogen Receptor-Associated Proteins: Possible Mediators of Hormone-Induced Transcription, Science. *264:* 1455-1458, 1994.

15.   Cavailles, V., Dauvois, S., Danielian, P. S., and Parker, M. G. Interaction of proteins with transcriptionally active estrogen receptors, Proc. Natl. Acad. Sci. USA. *91:* 10009-10013, 1994.

16.   Hanstein, B., Eckner, R., DiREnzo, J., Halachmi, S., Liu, H., Searcy, B., Kurokawa, R., and Brown, M. p300 is a component of an estrogen receptor coactivator complex, Proc. Natl. Acad. Sci. USA. *93:* 11540-11545, 1996.

17.   Cavailles, V., Dauvois, S., L'Horset, F., Lopez, G., Hoare, S., Kushner, P. J., and Parker, M. G. Nuclear factor RIP140 modulates transcriptional activation by estrogen receptor, EMBO J. *14:* 3741-3751, 1995.

18.   Lee, J. W., Ryan, F., Swaffield, J. C., Johnson, S. A., and Moore, D. D. Interaction of thyroid hormone receptor with a conserved transcriptional mediator, Nature. *374:* 91-94, 1995.

19.   Onate, S. A., Tsai, S. Y., Tsai, M.-J., and O'Malley, B. W. Sequence and characterization of a coactivator for the steroid hormone receptor family, Science. *270:* 1354-1357, 1995.

20.   Hong, H., Kohli, K., Trivedi, A., Johnson, D. L., and Stallcup, M. R. GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors, Proc. Natl. Acad. Sci. USA. *93:* 4948-4952, 1996.

21.   Voegel, J. J., Heine, M. J. S., Zechel, C., Chambon, P., and Gronemeyer, H. TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors, EMBO J. *15:* 3667-3675, 1996.

22.   Chakravarti, D., LaMorte, V. J., Nelson, M. C., Nakajima, T., Schulman, I. G., Juguilon, H., Montminy, M., and Evans, R. M. Role of CBP/p300 in nuclear receptor signalling, Nature. *383:* 99-103, 1996.

23.   Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X.-Y., Sauter, G., Kallioniemi, O.-P., Trent, J. M., and Meltzer, P. S. AIB1, a Steroid Receptor Coactivator Amplified in Breast and Ovarian Cancer, Science. *277:* 965-968, 1997.

24.   Migliaccio, A., Domenico, M. D., Green, S., deFalco, A., Kajtaniak, E. L., Blasi, F., Chambon, P., and Auricchio, F. Phosphorylation on Tyrosine of in Vitro Synthesized Human Estrogen Receptor Activates Its Hormone Binding, Mol. Endo. *3:* 1061-1069, 1989.

25.   Auricchio, F., Migliaccio, A., Domenico, M. D., and Nola, E. Oestradiol stimulates tyrosine phosphorylation and hormone binding activity of its own receptor in a cell-free system, EMBO. *6:* 2923-2929, 1987.

26.   Arnold, S. F., Obourn, J. D., Jaffe, H., and Notides, A. C. Serine 167 Is the Major Estradiol-Induced Phosphorylation Site on the Human Estrogen Receptor, Mol. Endo. *8:* 1208-1214, 1994.

27. Hunter, T. and Karin, M. The regulation of transcription by phosphorylation, Cell. *70:* 375-387, 1992.

28. Luo, K., Hurley, T. R., and Sefton, B. M. Cyanogen Bromide Cleavage and Proteolytic Peptide Mapping of Prteins Immobilized to Membranes, Methods in Enzymology. *201:* 149-152, 1991.

29. Boyle, W. J., Geer, P. V. D., and Hunter, T. Phosphopeptide Mapping and Phosphoamino Acid Analysis by Two-Dimentional Deparation on Thin-Layer Cellulose Plates, Methods in Enzymology. *201:* 110-149, 1991.

30. Sullivan, S. and Wong, T. W. A Manual Sequencing Method for Identification of Phosphorylated Amino Acids in Phosphopeptides, Analytical Biochemistry. *197:* 65-68, 1991.

31. Kushner, P. J., Hort, E., Shine, J., Baxter, J. D., and Greene, G. L. Construction of Cell Lines that Express High Levels of the Human Estrogen Receptor and Are Killed by Estrogens, Molecular Endocrinology. *4:* 1465-1473, 1990.

32. Kumar, V. and Chambon, P. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer, Cell. *55:* 145-156, 1988.

33. Lees, J. A., Fawell, S. E., White, R., and Parker, M. G. A 22-Amino-Acid Peptide Restores DNA-Binding Activity to Dimerization-Defective Mutants of the Estrogen Receptor, Mol. Cell Biol. *10:* 5529-5531, 1990.

34. Ince, B. A., Zhuang, Y., Wrenn, C. K., Shapiro, D. J., and Katzenellenbogen, B. S. Powerful Dominant Negative Mutants of the Human Estrogen Receptor, Journal of Biological Chemistry. *268:* 14026-14032, 1993.

35. Litchfield, D. W. and Luscher, B. Casein kinase II in signal transduction and cell cycle regulation, Molecular and Cellular Biochemistry. *127/128:* 187-199, 1993.

36. Arnold, S. F., Obourn, J. D., Jaffe, H., and Notides, A. C. Phosphorylation of the Human Estrogen Receptor by Mitogen-activated Protein Kinase and Casein Kinase II: Consequence on DNA Binding, J. Steroid Biochem. Molec. Biol. *55:* 163-172, 1995.

FIGURE LEGENDS

Figure 1. Sequence alignment of serine 559 phosphorylation site. Edman degradation indicated serine 559 (underlined) of hER is the putative site of hormone dependent phosphorylation. This region of the receptor shows weak homology among the known ER sequences shown.

Figure 2. *In vitro* kinase analysis of S559A mutant shows loss of hormone-dependent phosphorylation. Serine 559 of hER was mutated to alanine in GST-LBD and expressed in *E.coli. In vitro* kinase assays were performed with wild type (WT) and the mutant (S559A). Lane 3 shows phosphorylation of the WT in the presence of estradiol (E2). No phosphorylation is seen with the S559A mutant in lane 4.

Figure 3. Estradiol stimulates phosphorylation of hER in CHO-ER cells. CHO-ER cells were metabolically labeled with [^{32}P]-orthophosphate. Cells were stimulated with 10 nM estradiol (E2) or ethanol vehicle (---). hER was immunoprecipitated from cell lysates and subjected to SDS-PAGE on a 10% polyacrylamide gel followed by blotting to nitrocellulose membranes. Autoradiography of membranes shows strong stimulation of phosphorylation for hER in the presence of E2 (arrow).

Figure 4. Phosphotryptic peptide mapping of *in vitro* and *in situ* phophosphorylated ER. GST-LBD and hER bands were excised from nitrocellulose membranes and subjected to tryptic digestion. Eluted peptides were separated in two dimensions on thin layer cellulose (see text). Comparison of autoradiographs for phosphorylated GST-LBD and hER show overlapping phosphopeptides (arrows).

Figure 5. RP-HPLC analysis of phosphopeptides show similarity but not identity. Overlapping peptides were extracted from thin layer cellulose plates and applied to a C18 column. Peptides were eluted by RP-HPLC using a 3-45% acetonitrile gradient over 42 minutes. Fractions were monitored by Cerenkov counting. A) *In vitro* and *in situ* peptides elute at similar

gradient positions. B) The two peaks from (A) were pooled, lyophylized, and reapplied to the C18 column. Cerenkov counting of elutions indicates the peptides are not identical.

Figure 6. Amino acids 500-520 of hER are involved in kinase binding. Increasing amounts of the peptide encompassing amino acids 500-520 of hER were added simultaneously with kinase extracts and the ability of the peptide to block kinase activity was analyzed by *in vitro* kinase assays (lanes 1--8). Quantitation of phosphorylated receptor bands (AMBIS) is shown below indicating that the peptide is able to block kinase activity when present in a large stoichiometric excess. When peptide is preincubated with the kinase extract (see text for details), it is able to block activity (lane 9). When preincubated with immobilized LBD, the peptide does not block kinase activity (lane 10).

Figure 7. Serine 559 is not required for recruitment of the kinase to the receptor.
hER(297-555) affinity columns were prepared by immunoprecipitation. *In vitro* kinase assays (lanes 1 and 2) indicate the kinase activity is not able to phosphorylate this ER truncation mutant. Unbound proteins were analyzed for kinase activity by *in vitro* kinase assays using GST-LBD in the presence of E2 (lanes 4 and 5, in duplicate). The truncation mutant is able to deplete purified extracts of the kinase activity. Columns were prepared as in lane 2 (in duplicate) and ER-associated proteins were eluted with 500 mM NaSCN. Eluted samples were reapplied to GST-LBD columns in the presence of E2 and *in vitro* kinase assays were performed (lanes 6 and 7). A longer exposure indicates that the kinase activity can be eluted from columns containing the truncation mutant. Therefore, the truncation mutant does indeed retain the ability to bind the kinase.

Figure 8. Casein kinase 2 (CK2) is present in HeLa cell extracts, but not purified kinase fractions. A) Partially purified kinase extracts from HeLa cells were applied to GST-LBD columns in the absence or presence of E2. Associated proteins were eluted with Laemmli sample buffer and separated by SDS-PAGE. Gels were blotted to nitrocellulose and probed with anti-CK2 antibodies. The positive control shows the expected position of CK2. CK2 does not appear to be present in purified kinase extracts. B) Sucrose density gradient fractions from HeLa cell

extracts were collected and further separated by SDS-PAGE on 10% gels (lanes 1-12). Proteins were transferred to nitrocellulose and probed with the anti-CK2 antibody as before (+ lane is positive control). CK2 protein is found in fractions 7-9, distinct from where the kinase activity is found (fractions 2-4).

Figure 9. 4-hydroxytamoxifen exchange with estradiol causes the release of the kinase. A) GST-LBD/kinase complexes were preassembled in the presence of E2. Increasing amounts of OHT (0, 1X, 5X, 10X in lanes 1-4) were incubated with columns followed by analysis by *in vitro* kinase assays (see text). Autoradiography demonstrates that 10 fold excess OHT is able to completely abolish kinase activity. B) 10X OHT elutions were treated with charcoal and reapplied to GST-LBD columns in the presence of E2. *In vitro* kinase assays were performed. Lanes 1 and 2 show activity which remained on columns after OHT treatment. Lane 3 is a control sample with no OHT. Lanes 4 and 5 show activity eluted from the column in lanes 1 and 2 and reapplied to GST-LBD.

Figure 10. Kinase purification scheme. The kinase activity was purified from HeLa cell pellets as described.

Figure 11. Silver stain analysis of purified kinase activity suggests the kinase is part of a complex of 5 proteins. HeLa cell extracts were subjected to purification as described. The resultant elutions were analyzed by SDS-PAGE on a 6% polyacrylamide gel followed by silver staining. Lanes 1 and 2 show proteins remaining on affinity columns after OHT elution. The strong bands are GST-LBD. Lanes 3 and 4 represent flowthrough from affinity columns. Lanes 5 and 6 show OHT elution profiles. Some GST-LBD is found in elutions (asterisks). Five stoichiometrically-bound proteins recognize GST-LBD only in the presence of E2 and are eluted with OHT (arrows).
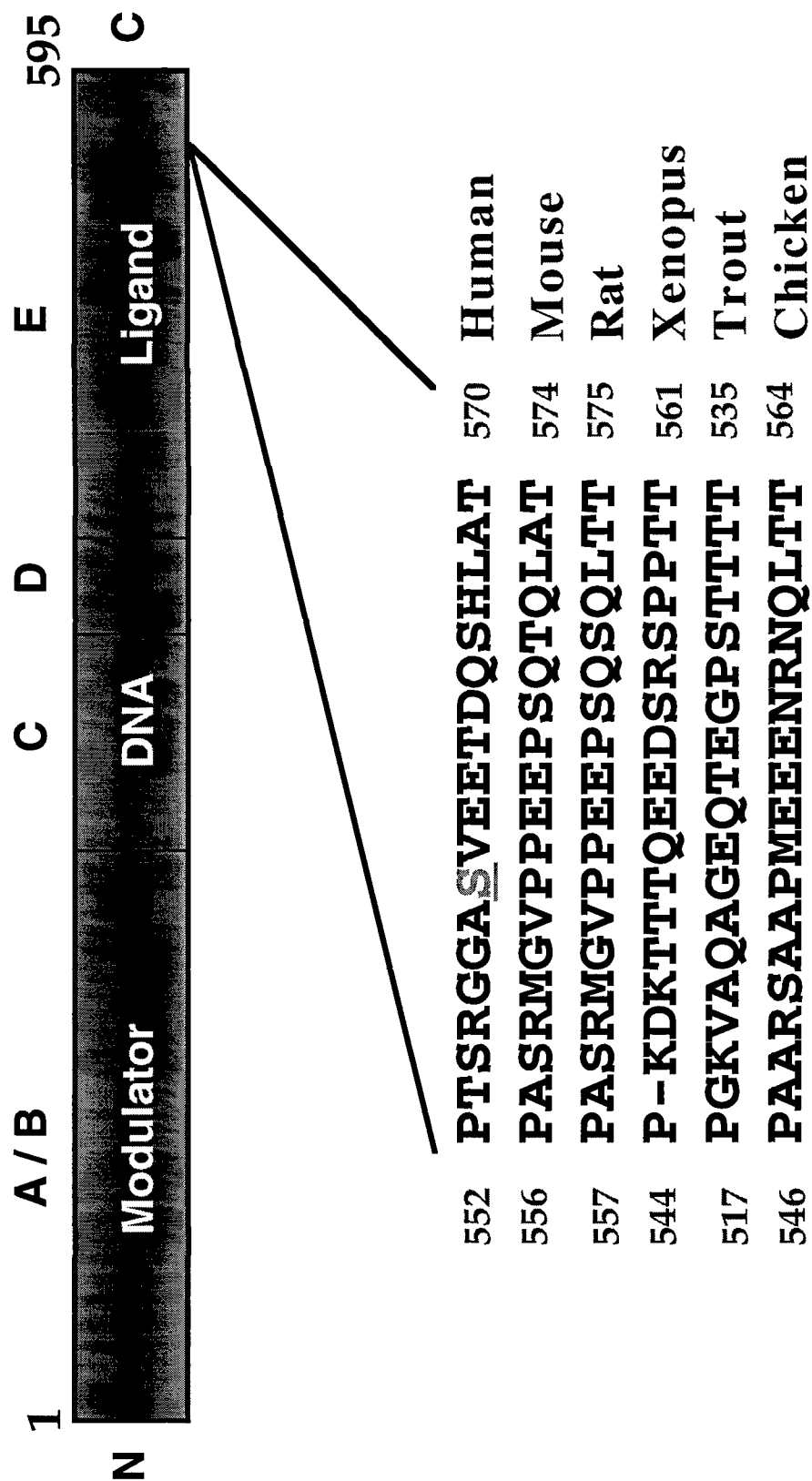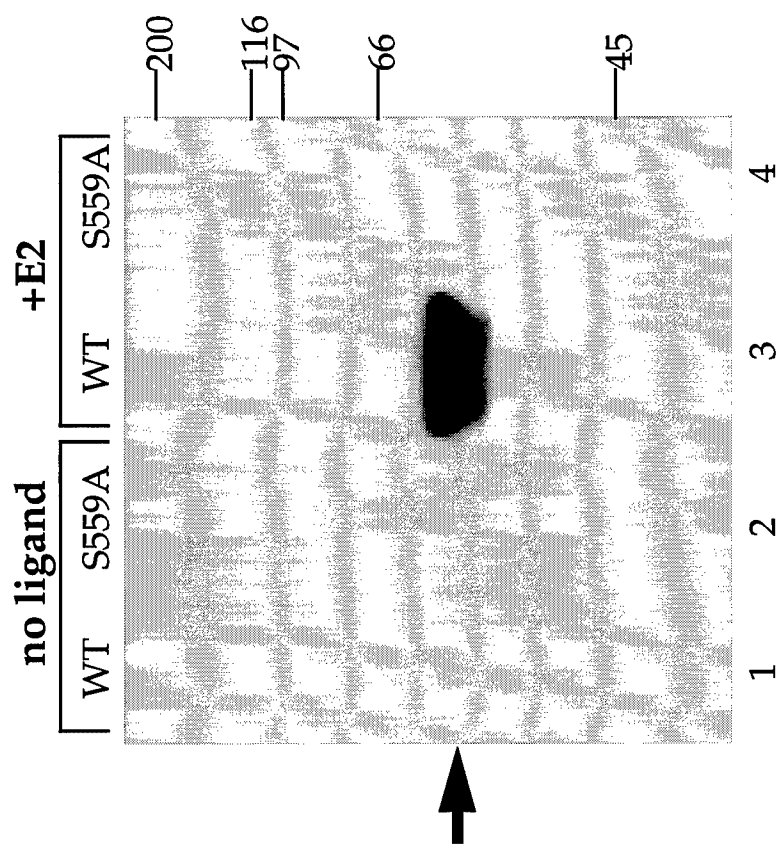
Figure 1.

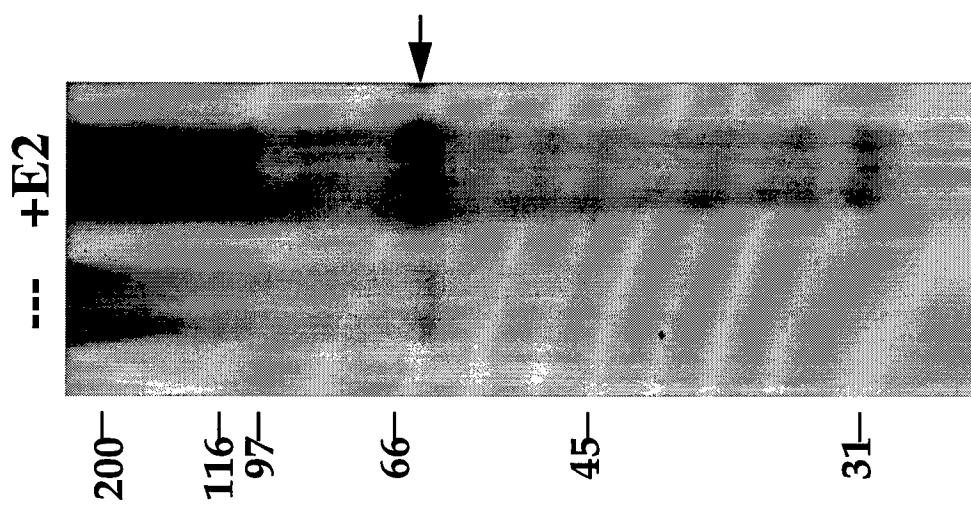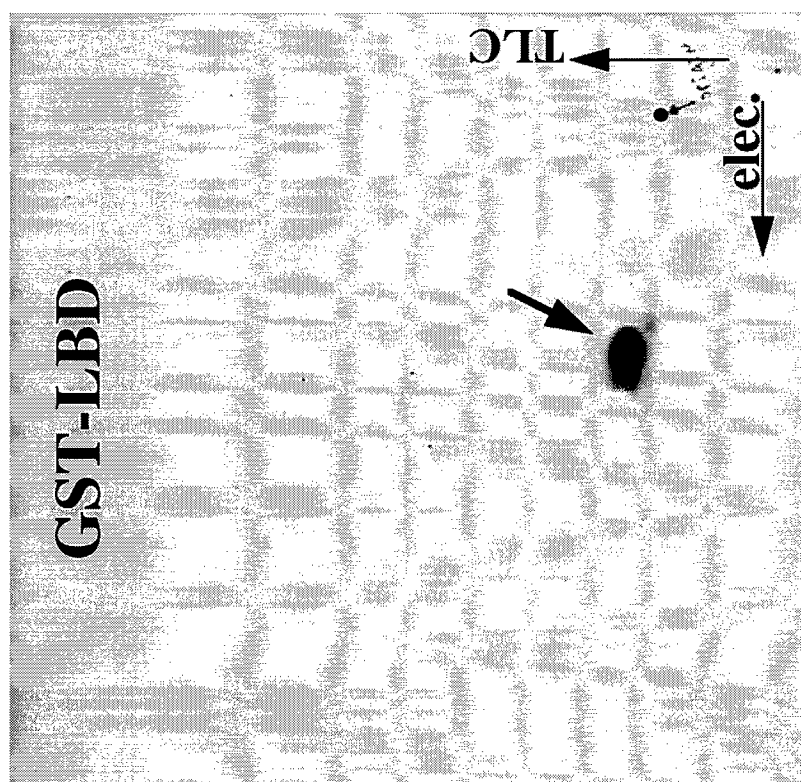|					
552	PTSRGGA<u>S</u>VEETDQSHLAT	570	Human
556	PASRMGVPPEEPSQTQLAT	574	Mouse
557	PASRMGVPPEEPSQSQLTT	575	Rat
544	P–KDKTTQEEDSRSPPTT	561	Xenopus
517	PGKVAQAGEQTEGPSTTT	535	Trout
546	PAARSAAPMEEENRNQLTT	564	Chicken

Figure 2.

Figure 3.

31
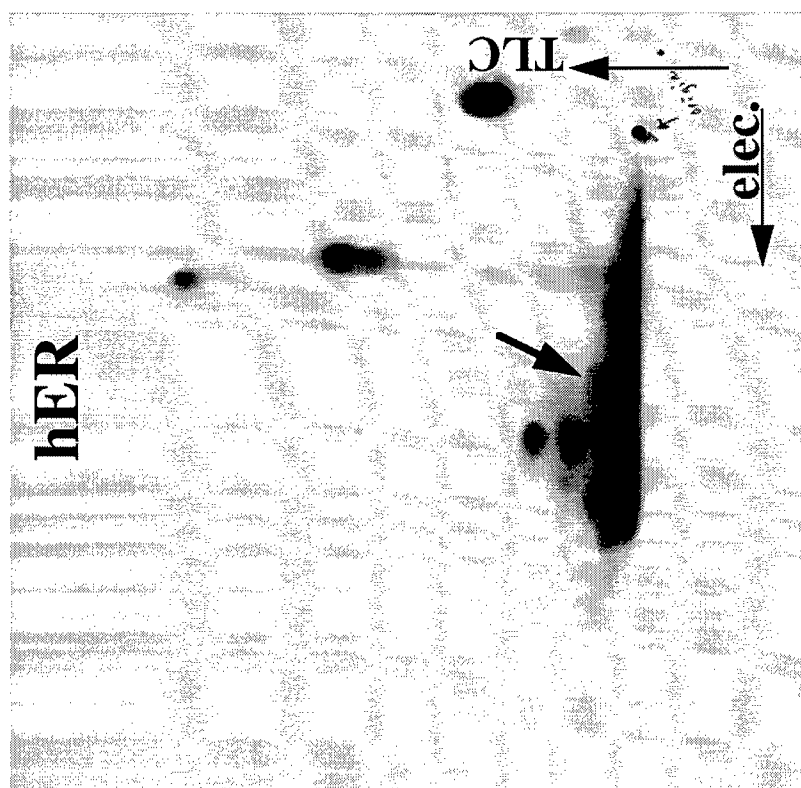
Figure 4.

A.

B.

Figure 5.

Figure 6.

Figure 7.

longer exposure

reapply elution

Depletion

ER(297-566) control

ER(297-555)

lane#

35

A.

positive control

--- +E2

B.

97

66

45

31

frac.# 1 2 3 4 5 6 7 8 9 10 11 12 +

Figure 8.

A.

Loss of IVK activity
with increasing [OHT]

B.

Reconstitution of IVK activity after
elution with OHT
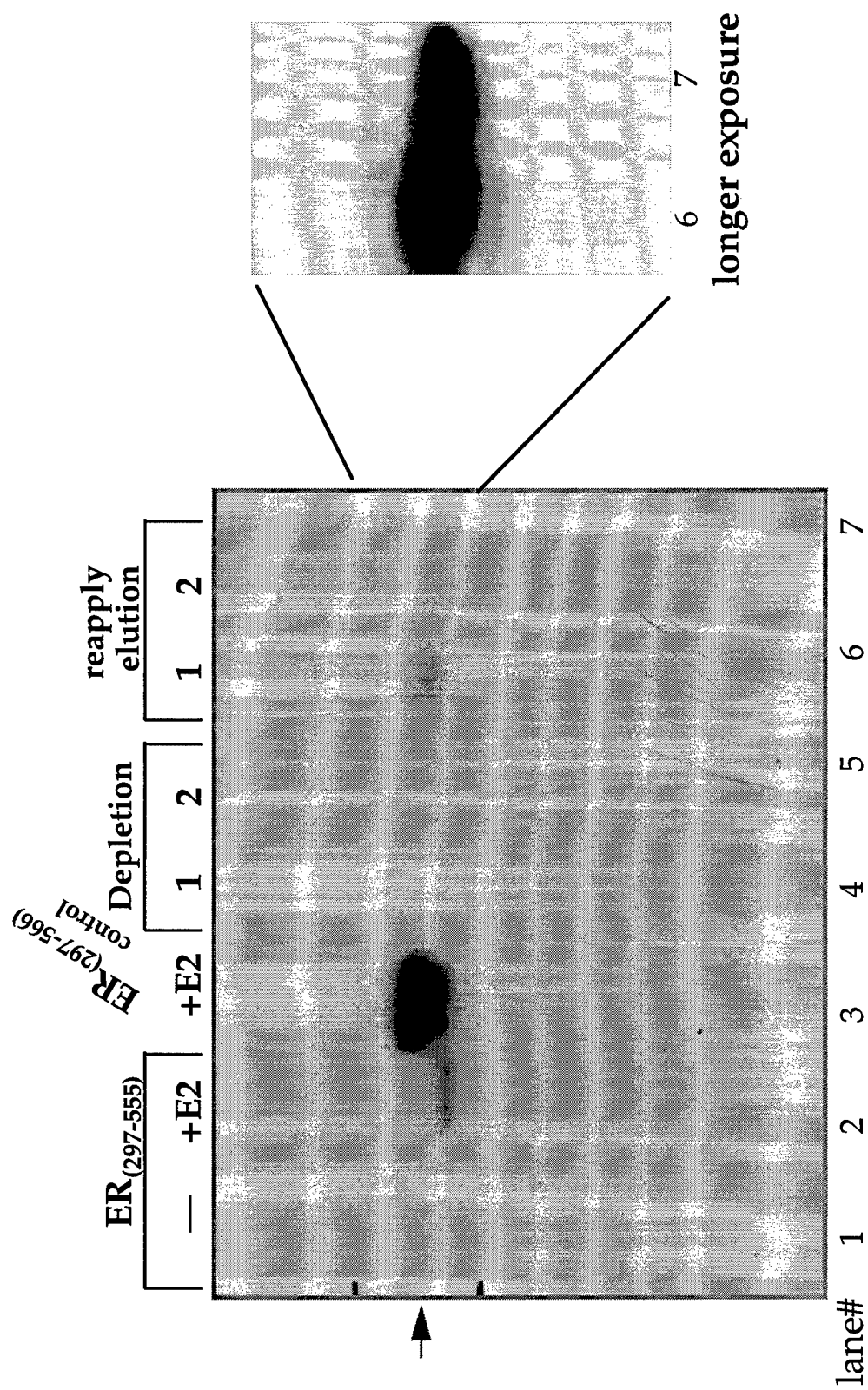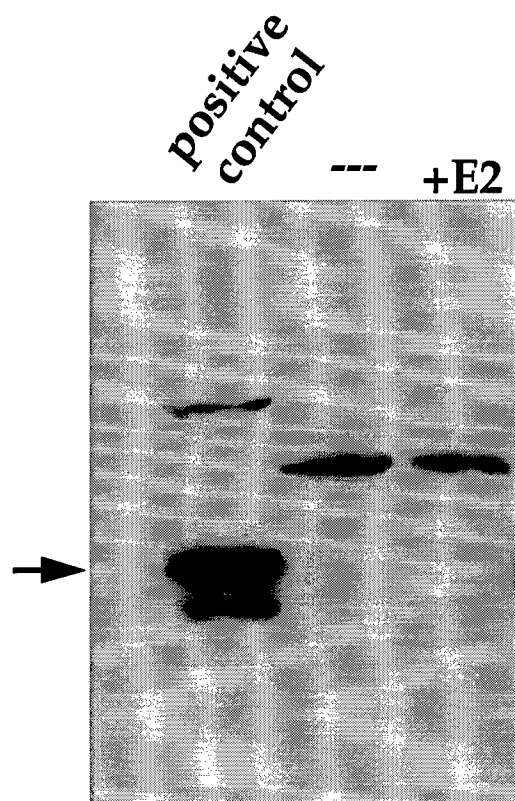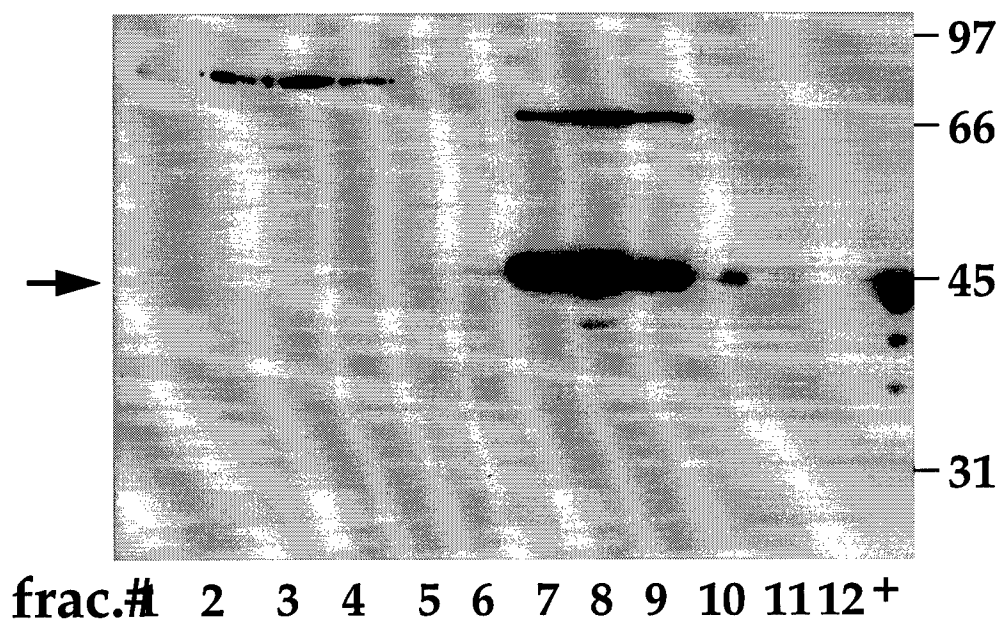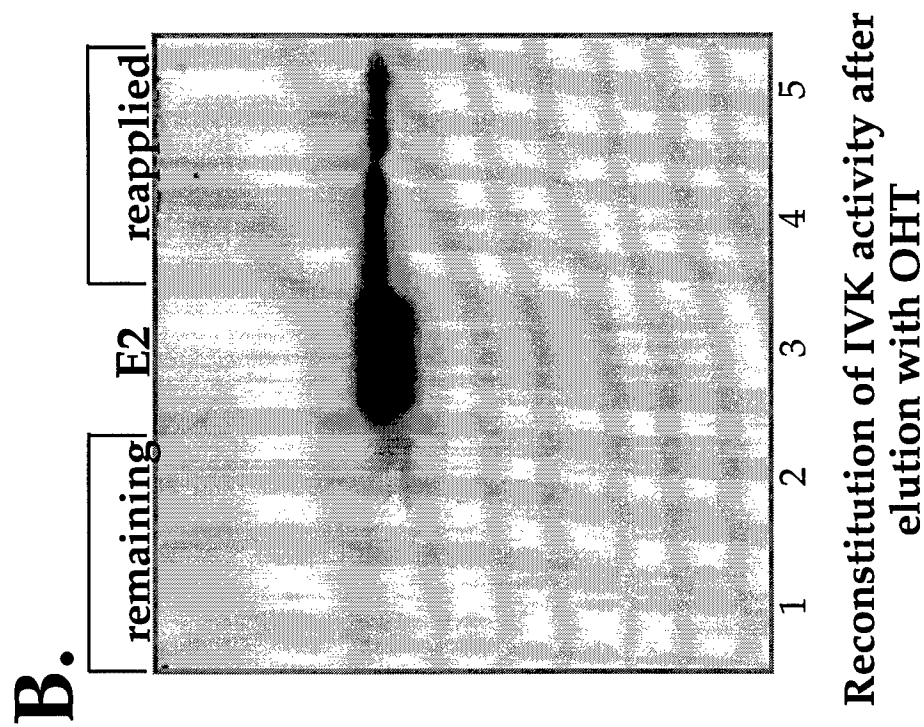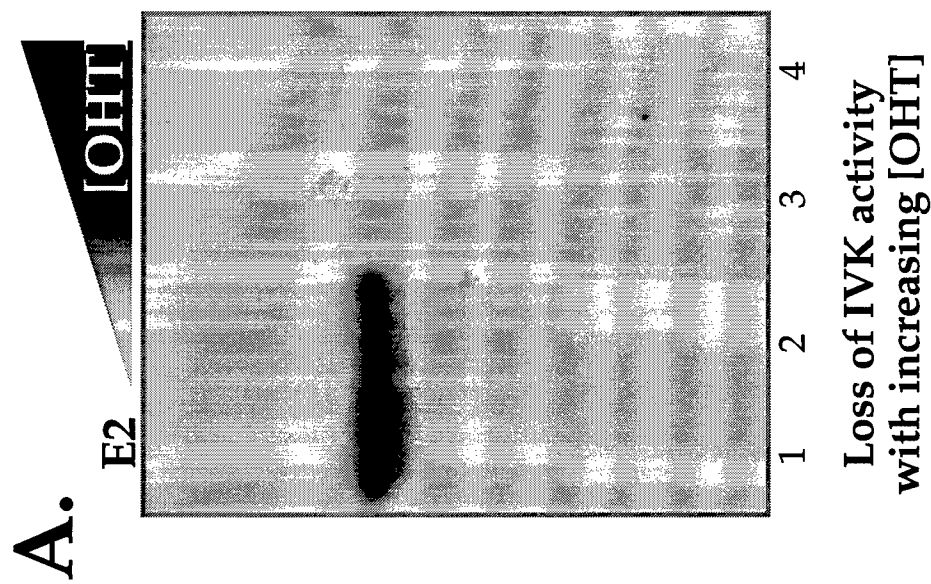
Figure 9.

Purification Scheme

Whole cell extracts from
mammalian cell culture

↓

10-30% sucrose density gradient

↓

ion exchange on MonoQ column

↓

clearance on ER-LBD column (no ligand)

↓

bind flow through to columns containing
GST-LBD + **E2**

↓

high salt washes

↓

E2/OHT ligand exchange to elute activity

↓

examine proteins by silver stain

Figure 10.

Figure 11.

ANNUAL REPORT FOR GRANT NO. DAMD17-94-J-4228

Appendix

Three copies each of two publications and one manuscript that has been accepted for publication in the *Journal of Steroid Biochemistry and Molecular Biology* (September, 1997 issue)

Estrogen Receptor Structure and Function in Breast Cancer Cells

Geoffrey L. Greene, Vandana Sharma, and Lin Cheng

Estrogen target tissues in the human include the uterus, mammary gland, ovary, vasculature, bone, brain, pituitary, and liver. A characteristic of these tissues is that they contain at least one intracellular recept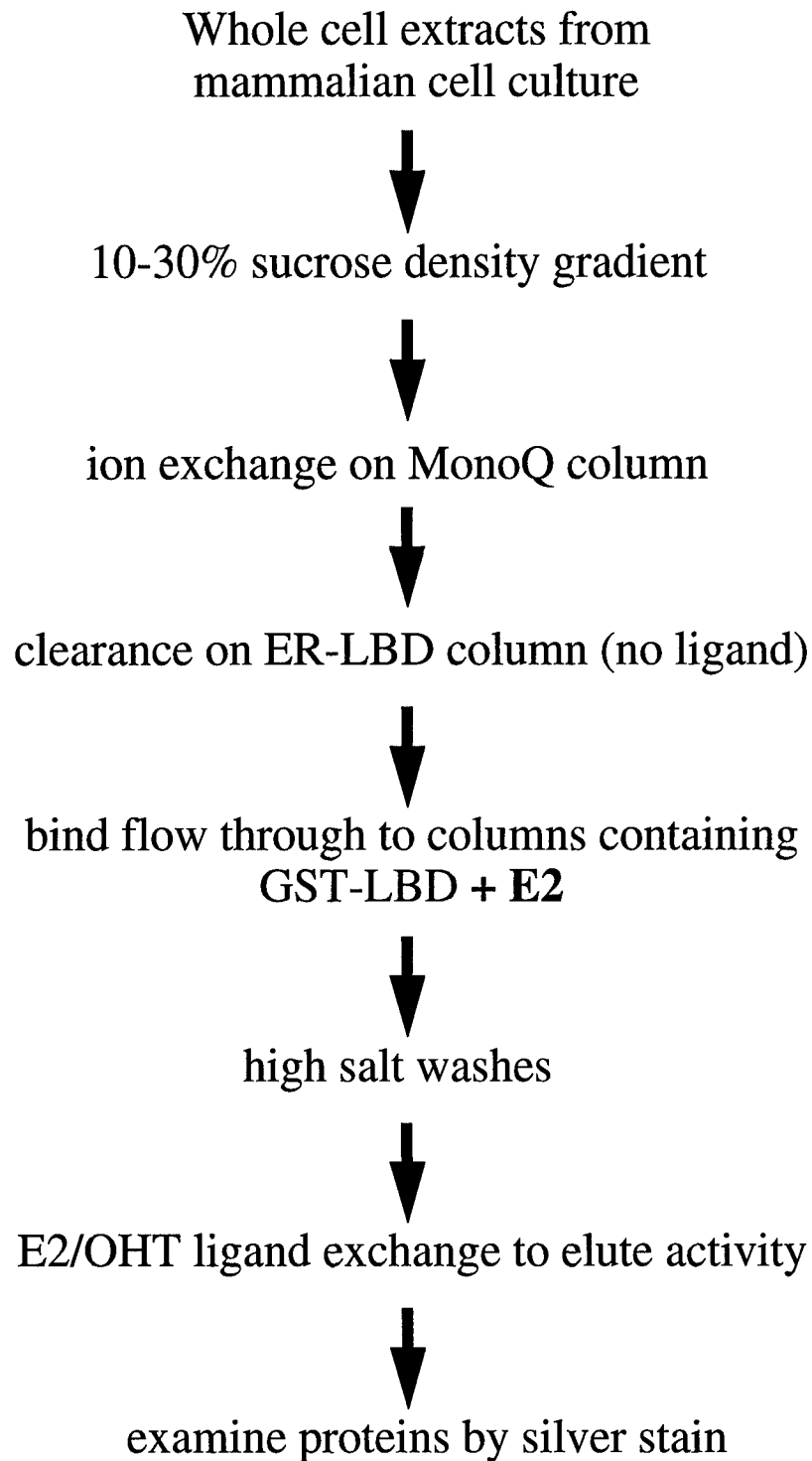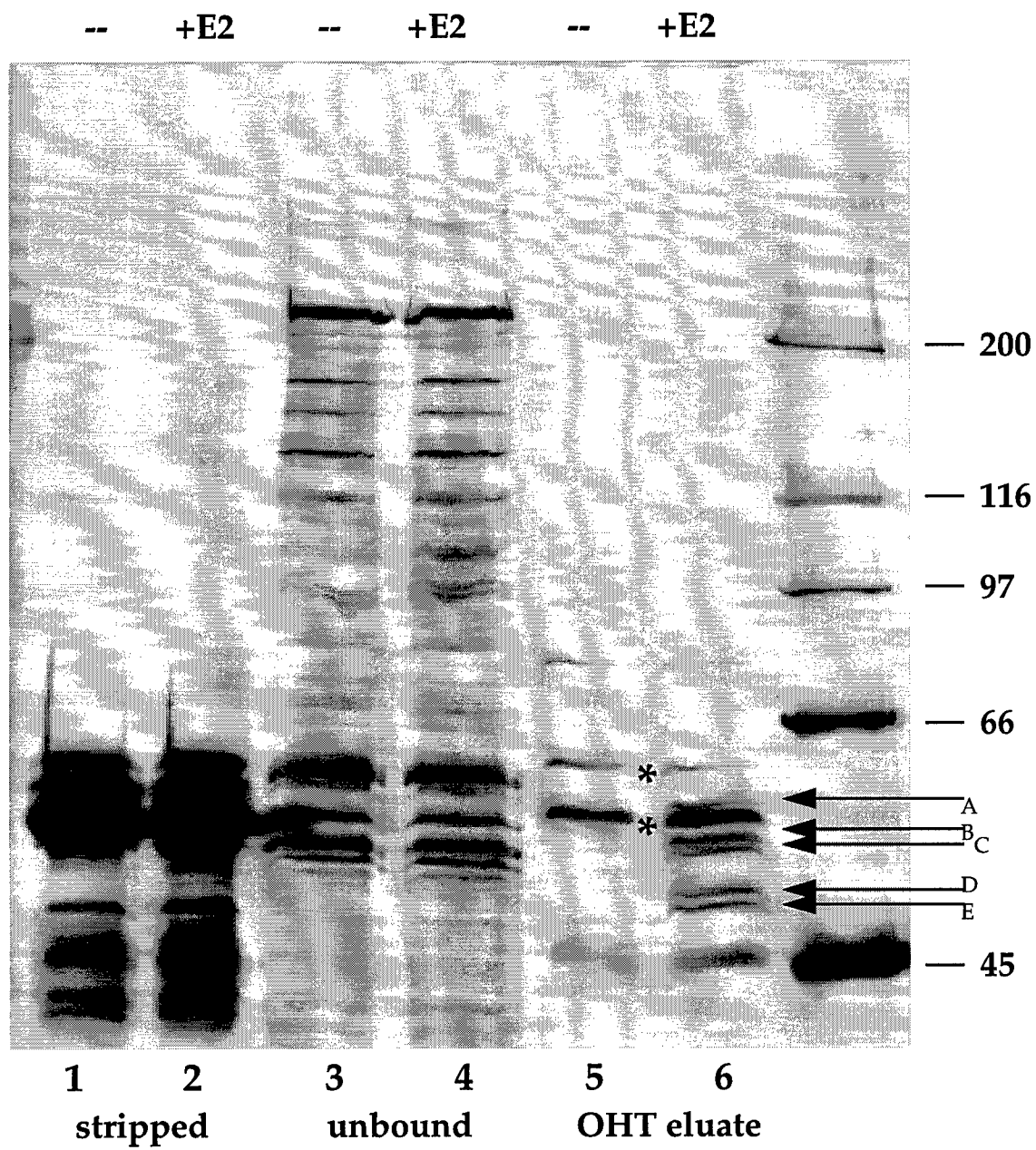or protein for estrogen. The existence of an estrogen receptor (ER) was first proposed more than thirty years ago by Elwood Jensen[1] and Jack Gorski.[2] However, despite numerous advances in our understanding of the structure and function of nuclear receptors,[3,4] the molecular mechanisms by which female steroid hormones and their antagonists regulate cellular proliferation in hormone responsive tissues and cancers are still poorly defined. Because ER is a key mediator of estrogen-dependent growth, knowledge of both the structure and action of this protein is essential to elucidate the origins, progression, and control of hormone-dependent cancers. We and others are actively studying multiple aspects of ER action, including the role of phosphorylation and cross talk with other signalling pathways, the roles of ER-associated accessory or co-regulator proteins, the identification of genes that are differentially regulated by estrogen agonists and antagonists, and the detailed structural requirements for ligand binding to ER, especially in regard to discrimination between agonists and antagonists such as tamoxifen. The picture has become more complicated by the recent discovery of a second receptor protein for estrogen, designated ERβ.[5] However, ERβ does not appear to be expressed in mammary tissue and is significantly less abundant in the uterus than ER (ERα). The studies described here relate only to the action of ER-α, which will be referred to as ER.

Estrogen Receptor Action

Estrogen action is initiated by entry of the hormone into a target cell, generally thought to be by passive diffusion, where it interacts with a predominantly

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nuclear ER[6] that is in dynamic equilibrium between the cytoplasm and nucleus.[7] A complex sequence of events occurs at this point, including dissociation of one or more heat shock proteins from ER, as well as increased ER phosphorylation, dimerization, and interaction both directly and indirectly with hormone response elements (EREs) to increase or repress specific target genes (Figure 1). The resulting changes in mRNA levels constitute the hormone response for a target tissue. A tissue-specific increase or decrease in the expression of a number of genes in reproductive tract tissues and their cancers is known to occur, including c-fos, c-myc, TGFα, TGFβ, IGF-1, progesterone receptor, cyclin D1, and bcl-2, to name just a few. Clearly, diverse pathways and cellular processes are sensitive to estrogen and ER action.

The ER is a member of the nuclear hormone receptor family, which is a rather large and growing family of transcription factors that includes not only the steroid receptors, but a number of orphan receptors as well. Although several dozen members of this family have been cloned, target genes and ligands have been identified for only a limited number of these receptors. A characteristic of the estrogen receptor, as well as other members of the nuclear receptor family, is that the molecule can be divided into three major functional regions (Figure 2): a DNA binding domain that defines the family, a hormone binding
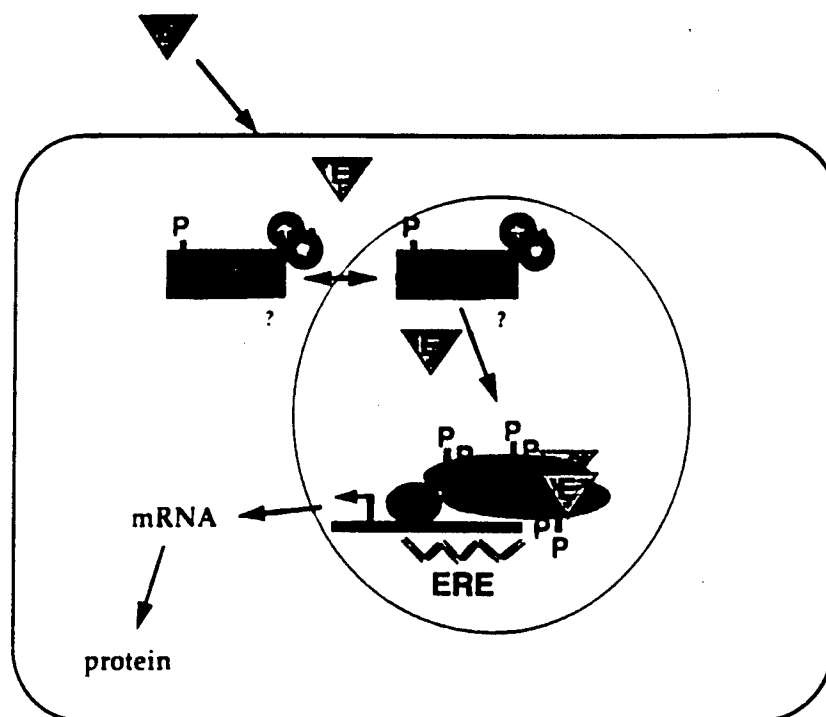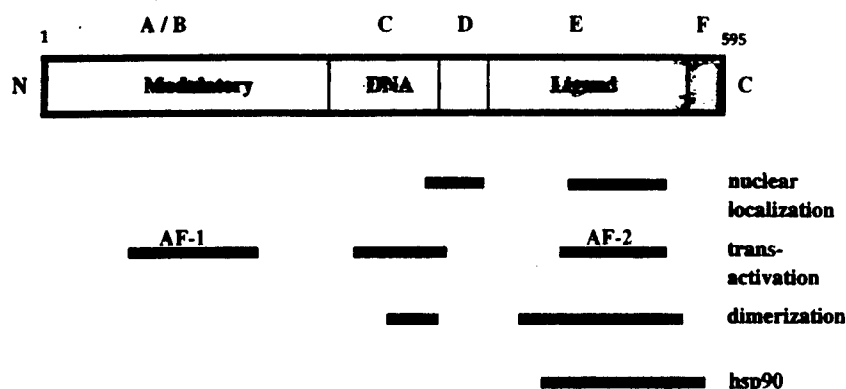

Figure 1. Model of estrogen action in a hormone responsive cell.

Figure 2. Schematic representation of the human estrogen receptor showing several functional domains and associated activities.

domain, and a modulatory region, which varies considerably in length among members of the family. Within these domains are multiple subfunctions. For example, the amino terminal modulatory region contains a transcriptional activating function referred to as AF-1, whereas the ligand binding domain contains another transcriptional activating function, AF-2. The ligand binding domain also participates in homodimerization and/or heterodimerization, as does the DNA binding domain, as well as in the interaction of ER with other proteins, as described below. In addition, nuclear translocation signals are present in the hinge region located between the DNA-binding domain and the ligand-binding domain. Thus, many activities are carried out by nuclear receptors; the molecular details and consequences of these activities are the focus of numerous investigations.

The estrogen receptor interacts with a number of other proteins both before and after activation by hormone. The identities and functions of some of these receptor-associated proteins are beginning to emerge. At least three members of the heat-shock protein family have been identified as putative accessory proteins by virtue of their association with several receptors in vitro. One of these, hsp90, has been implicated in the stabilization of the inactive form of receptors for glucocorticoids (GR), progestins (PR) and estrogens (ER).[8] Estrogen receptors can also interact with other transcription activators (*e.g.*, AP-1)[9] as well as various co-regulators (*e.g.*, CAMP response element binding protein and SRC-1)[4,10,11] and members of the basal transcription apparatus such as TFIIB. In addition, the intersection of peptide growth factor signal pathways with ER[12] and other members of the nuclear receptor family[13] has recently been implicated in the modulation of the activities of nuclear receptors. This cross-talk may involve both tyrosine[14] and serine[15] kinase activity and probably phosphatase activity as well. The estrogen receptor, as well as other transcription factors, are phosphoproteins and are phosphorylated or

dephosphorylated as part of their action. Thus, an understanding of the intersection of these pathways is essential to understanding steroid hormone action.

The Use of RNA Differential Display to Identify Estrogen Agonist/Antagonist-Responsive Genes in Breast Cancer Cells

Breast cancer is a complex, systemic disease, and tumor growth, progression, and metastasis depend on the intersection of multiple signal pathways, some of which are either directly or indirectly regulated by estrogens in hormone dependent tumors. During tumor progression, many autocrine and paracrine factors stimulate proliferation and recruitment of stromal tissue and blood supply to the expanding tumor. Some of these factors, such as TGF-α, TGF-β, c-myc, hsp27, cyclin D1, bcl-2, progesterone receptor, and one or more angiogenic factors, such as pleiotrophin,[16,17] are regulated by ER and are presumably part of the proliferative or invasive response. However, the sequential and/or concurrent molecular events that are required for net expansion of hormone sensitive tumors remain to be elucidated. In addition, one of the major current challenges is to explain the differential action of estrogens and estrogen antagonists among hormone sensitive tissues.

To identify novel genes that are selectively regulated by different estrogenic ligands, we used the technique of RNA differential display to identify several partial cDNA clones that are differentially regulated by estradiol (E2), 4-hydroxytamoxifen (OH-T, a partial antagonist) and ICI 182,780 (ICI, a complete antagonist), in the estrogen-sensitive MCF-7 breast cancer cell line. RNA differential display is a polymerase chain reaction-based technique that allows one to visualize and isolate messenger RNAs that are being expressed at any given time in a tissue or in a cell. By amplifying the pool of mRNAs from a tissue or a cell that has been treated differentially with estrogen agonists or antagonists, one can determine any differences in RNA expression that occur. One would predict that for some genes, estradiol will selectively induce the expression of a particular messenger RNA, which would not be seen on the sequencing gel in response to 4-hydroxytamoxifen. Likewise, one might find selective suppression of a gene. In fact, both phenomena were observed. An advantage of this technique is that it is possible to isolate, clone, and sequence the corresponding cDNAs directly from selected bands on the differential gel display.

For one 300 bp cDNA, selective suppression by estradiol was observed on the differential display screen. Nucleotide sequence analysis and Northern blot hybridization patterns of this clone indicated that it was human monocyte chemoattractant protein-1 (MCP-1).[18,19] Human monocyte chemoattractant protein-1 is a cytokine produced by many cell types in response to mitogenic stimulation. It is induced by a number of factors, including TNF-α, and is normally expressed in monocytes, fibroblasts, vascular endothelium, and vascular

smooth muscle cells. It was not previously known to be estrogen sensitive, and was selectively downregulated in MCF7 and T47D cells by estradiol but not by 4-hydroxytamoxifen or ICI 182,780. Monocytes are frequently found in close association with tumors, and are postulated to play an active role in the local tumor environment. Therefore, the specific modulation of a monocyte chemoattractant could be a significant component of tumor biology. Interestingly, it was recently reported that the related chemokine JE/MCP-2 is suppressed by estrogen in murine macrophages.[20] A number of pathological conditions are accompanied by increased MCP-1 expression, including malignant gliomas and fibrosarcomas, atherosclerosis, and others. Thus, it is very interesting that this gene product, which is involved in regulation of monocyte or macrophage chemotaxis, also appears to be under estrogen regulation in human breast cancer cells.

A second transcript, which was specifically induced in MCF-7 cells by estradiol and not by 4-hydroxytamoxifen or ICI 182,780, represents a 7.5 kb mRNA species recently identified as the MN1 gene. Two groups recently reported the existence of the MN1 gene (98% sequence match with our 700 bp cDNA in the 3' untranslated region). In one study, MN1 was disrupted by a balanced translocation (4;22) in a meningioma.[21] In a separate study, translocation (12;22) in an acute myeloid leukemia resulted in a fusion of the TEL gene (12p13), a member of the ETS family of transcription factors, to the MN1 gene on 22q11. The existence of both TEL-MN1 and MN1-TEL fusion proteins was predicted. Because the MN1 gene appears to be transcriptionally induced by estrogens in MCF-7 cells, it may also be regulated by estrogens in normal mammary tissue. Thus, the RNA display technique is likely to reveal novel mechanistic aspects of the differential regulation of proliferation and other specific cellular responses by estrogens and their antagonists. Such data may also prove useful for the treatment of breast cancer, osteoporosis, and heart disease in women.

Structural Studies of Estrogen Receptors

Estrogen receptor activity is modulated by the binding of ligand, which is similar to other steroid receptors. What is unusual about ER is that it is so promiscuous in terms of ligand recognition. Some of these ligands derive from plants (phytoestrogens), and others are industrial products or byproducts (chlorophenotane and various phenols). Estradiol is the most abundant natural agonist for the estrogen receptor. In addition, the synthetic compound tamoxifen is widely used in the treatment of breast cancer, both prophylactically as well as in adjuvant settings. An active metabolite of tamoxifen; 4-hydroxytamoxifen binds to ER with an affinity that is approximately the same as estradiol, although its structure looks quite different than estradiol (Figure 3). There are also molecules that have been referred to as complete estrogen antagonists, such as ICI 182,780,[22] which is an estradiol analog with an extended 7-alpha

OH

HO
Estradiol

N(CH₃)₂

O

HO
4-Hydroxytamoxifen

OH

HO
(CH₂)₉-SO-(CH₂)₃C₂F₆

ICI 182,780
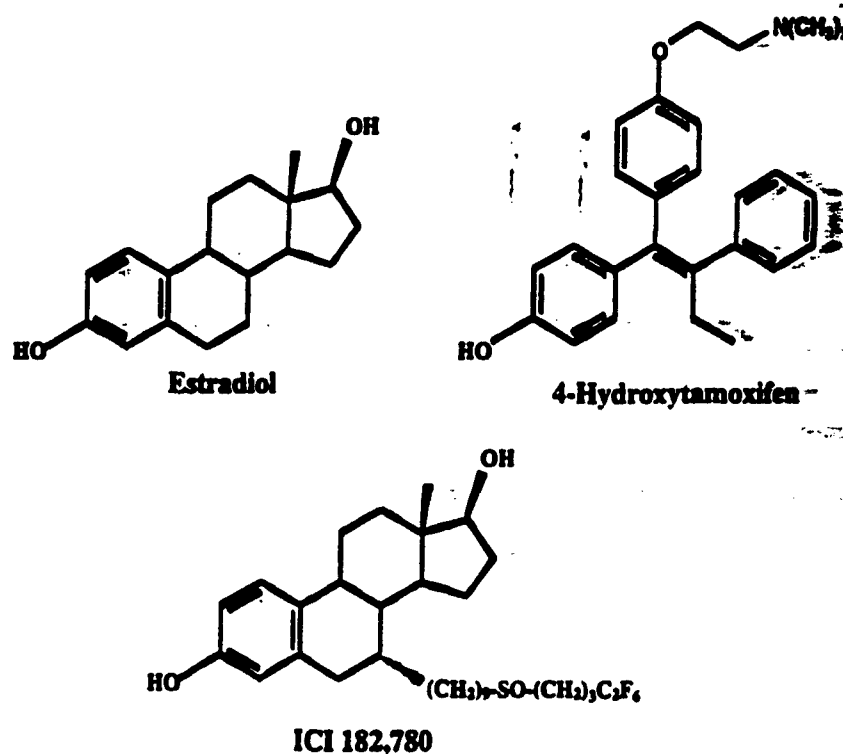
Figure 3. Structure comparison of estrogen agonists and antagonists.

hydrocarbon substituent that renders the estrogen receptor inactive by a mechanism that is not well defined. Estrogen antagonists can be grouped into two major categories. Those such as tamoxifen and 4-hydroxytamoxifen are partial antagonists, and their antagonism is both promoter-specific and tissue-specific. Thus, while 4-hydroxytamoxifen, or tamoxifen, is an antagonist in the breast, it is a partial agonist in bone, vasculature, and uterus. These molecules, when bound to ER, promote DNA binding but inhibit the AF-2 activating function, whereas compounds like the so called pure estrogen antagonist ICI-182,780, may block DNA binding by inhibiting dimerization.[23] They may alter nucleo-cytoplasmic shutting of ER,[24] or increase the degradation rate of ER.[25]

A key unresolved issue concerning ER is the difference in receptor structure and function when ER is bound to an agonist, partial antagonist, or a complete antagonist. Recent crystal structure data for RXR-α,[26] RAR-γ,[27] and TR-α₁,[28] with or without bound ligand, suggest that the ligand may determine the position and/or shape of several α-helices near the surface of the ligand binding domain, and especially the amphipathic helix (helix 12 in TR-α₁) that contains the AF-2 transcription activating domain. Interestingly, thyroid hormone agonists like tri-iodothyronine were found to be completely buried within the TR-α₁ ligand binding domain. Similarly, all trans-retinoic acid was

sealed within the corresponding hydrophobic pocket of RAR-γ, whereas in unliganded RXR-α, no such sealed pocket exists. Instead, the carboxyterminal helix (11 or 12, depending on the numbering system) that contains AF-2 is positioned away from the ligand binding domain, which leaves an open pocket for ligand. Thus, for at least two nuclear receptors, agonist ligands induce a conformation in which the amphipathic AF-2 α-helix is positioned over the ligand pocket and contributes to the hydrophobic interaction with ligand.

It is likely that the overall crystal structure of the agonist-occupied ER hormone binding domain will be very similar to the solved structures of agonist-bound RAR-γ, and TR-α₁. However, the important differences will be in the details and especially in how these subtle differences alter protein-protein interactions between ER and components of the transcription machinery. Thus, a knowledge of ER structure when it is occupied by ligands with differing biological properties will be essential for understanding the nature of tissue-specific agonism versus antagonism. Such information may also lead to the design of new ER ligands that will have selective positive, neutral, or negative effects in hormone sensitive tissues and cancers. Although it is not entirely clear whether rational drug design will be a useful application for such structural information, it is certainly a hope and goal for the future.

REFERENCES

1. Jensen E, Jacobson H: Basic guides to the mechanism of estrogen action. Recenti Progr Horm Res 1962; 18:387–414.
2. Toft DO, Gorski J: A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proc Natl Acad Sci U S A 1966; 55:1574–1581.
3. Mangelsdorf D, Thummel C, Beato M, *et al.*: The nuclear receptor superfamily: the second decade. Cell 1995; 83:835–839.
4. Horwitz K, Jackson T, Bain D, *et al.*: Nuclear receptor coactivators and corepressors. Mol Endocrinol 1996; 10:1167–1177.
5. Kuiper G, Enmark E, Pelto-Huikko M, *et al.*: Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 1996; 93:5925–5930.
6. King W, Greene G: Monoclonal antibodies localize estrogen receptor in the nucleus of target cells. Nature 1984; 307:745–747.
7. Guiochon-Mantel A, Lescop P, Christin-Maitre S, *et al.*: Nucleocytoplasmic shuttling of the progesterone receptor. EMBO J 1991; 10:3851–3869.
8. Smith D, Toft D: Steroid receptors and their associated proteins. Mol Endocrinol 1993; 7:4–11.
9. Pfahl M: Nuclear receptor/AP-1 interaction. Endocr Rev 1993; 14:651–658.
10. Onate SA, Tsai SY, Tsai MJ, *et al.*: Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 1995; 270:1354–1357.
11. Kamei Y, Xu L, Heinzel T, *et al.*: A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell 1996; 85:403–414.
12. Trowbridge-Ignar D, Nelson K, Bidwell M, *et al.*: Coupling of dual signaling pathways: epidermal growth factor action involves the estrogen receptor. Proc Natl Acad Sci U S A 1992; 89:4658–4662.
13. Mani S, Allen J, Clark J: Convergent pathways for steroid hormone- and neurotransmitter-induced rat sexual behavior. Science 1994; 265:1246–1249.

14. Pietras R, Arboleda J, Reese D, et al.: HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene 1995; 10:2435-46.

15. Kato S, Endoh H, Masuhiro Y, et al.: Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 1995; 270:1491-1494.

16. Riegel AT, Wellstein A: The potential role of the heparin-binding growth factor pleiotrophin in breast cancer. Breast Cancer Res Treat 1994; 31:309-14.

17. Zhang L, Rees MC, Bicknell R: The isolation and long-term culture of normal human endometrial epithelium and stroma. Expression of mRNAs for angiogenic polypeptides basally and on oestrogen and progesterone challenges. J Cell Sci 1995; 108:323-31.

18. Loetscher P, Seitz M, Clark-Lewis I, et al.: Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human CD4$^+$ and CD8$^+$ T lymphocytes. 1994; 8:1055-1060.

19. Yoshimura T, Yuhki N, Moore S, et al.: Human monocyte chemoattractant protein-1 (MCP-1). Full-length cDNA cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to mouse competence gene JE. FEBS Lett 1989; 244:487-493.

20. Frazier-Jessen M, Kovacs E. Estrogen modulation of JE/monocyte chemoattractant protein-2 mRNA expression in murine macrophages. J Immunol 1995; 154:1838-1845.

21. Lekanne Deprez R, Riegman P, Groen N, et al.: Cloning and characterization of MN1, a gene from chromosome 22q11, which is disrupted by a balanced translocation in a meningioma. Oncogene 1995; 10:1521-1528.

22. Weatherill PJ, Wilson APM, Nicholson RI, et al.: Interaction of the antioestrogen ICI 164,384 with the oestrogen receptor. J Steroid Biochem 1988; 30:263-266.

23. Fawell S, White R, Hoare S, et al.: Inhibition of estrogen receptor-DNA binding to the "pure" antiestrogen ICI 164,384 appears to be mediated by impaired receptor dimerization. Proc Natl Acad Sci U S A 1990; 87:6883-6887.

24. Dauvois S, White R, Parker M: The antiestrogen ICI 182780 disrupts estrogen receptor nucleocytoplasmic shuttling. J Cell Sci 1993; 106:1377-1388.

25. Gibson MK, Nemmers LA, Beckman WCJ, et al.: The mechanisms of ICI 164,384 antiestrogenicity involves rapid loss of estrogen receptor in uterine tissue. Endocrinology 1991; 129:2000-2010.

26. Bourguet W, Ruff M, Chambon P, et al.: Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-α. Nature 1995; 375:377-385.

27. Renaud J, Rochel N, Ruff M, et al.: Crystal structure of the RAR-γ ligand-binding domain bound to all-trans retinoic acid. Nature 1995; 378:681-689.

28. Wagner R, Apriletti J, McGrath M, et al.: A structural role for hormone in the thyroid hormone receptor. Nature 1995; 378:690-697.

Estrogen Receptor Affinity and Location of Consensus and Imperfect Estrogen Response Elements Influence Transcription Activation of Simplified Promoters

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We have examined the ability of estrogen receptor (ER) to bind and bend DNA fragments containing the *Xenopus laevis* vitellogenin A2 estrogen response element (ERE), which contains a palindromic, consensus ERE sequence, the *X. laevis* vitellogenin B1 ERE2, which contains a 1-bp mismatch in the 5′-end of the half-palindrome, and the human pS2 ERE, which contains a 1-bp mismatch in the 3′-end of the half-palindrome. ER binding induced a 65° bend in DNA fragments containing the consensus ERE, the vitellogenin B1 ERE2, or the pS2 ERE. However, ER affinity for the consensus ERE was 2-fold greater than for either the vitellogenin B1 ERE2 or the pS2 ERE. When Chinese hamster ovary (CHO) cells were transfected with reporter plasmids containing either the consensus ERE, the vitellogenin B1 ERE2, or the pS2 ERE separated from the TATA sequence by 2.6 helical turns, exposure to 10 nM 17β-estradiol increased transcription 12.7-, 2.4-, and 3.8-fold, respectively. Increasing the spacing between the ERE and TATA sequence to three helical turns decreased the ability of the consensus ERE to activate transcription by 55% and increased the ability of the pS2 ERE to activate transcription by 35% but had no significant effect on vitellogenin B1 ERE2 activity. Further increasing the distance between the ERE and TATA sequence to 3.6 helical turns restored the activity of promoters containing the consensus ERE and pS2 ERE but decreased the activity of the promoter containing the relatively weak vitellogenin B1 ERE2. These data support the idea that 1) the affinity of ER for the ERE, 2) the location of an ERE within the promoter, and 3) the magnitude and orientation of DNA bends induced by binding of ER or other proteins are important in transcription activation of estrogen-responsive genes. (Molecular Endocrinology 10: 694–704, 1996)

INTRODUCTION

The estrogen receptor (ER) is a member of a superfamily of ligand-activated transcription factors that play crucial roles in developmental progression and maintenance of normal reproductive cell function. The ER brings about changes in cellular function by binding to ligand and then interacting with a specific DNA sequence, the estrogen response element (ERE), to modulate transcription of estrogen-responsive genes (1, 2).

Estrogen-responsive genes typically contain one or more copies of the consensus ERE, a perfect palindromic sequence, or an imperfect ERE, which deviates from the consensus sequence by one or more nucleotides. The consensus ERE, which is found in the endogenous *Xenopus laevis* vitellogenin A2 gene (GGTCAnnnTGACC, Ref. 3) has been most extensively characterized, both in its ability to interact with ER and to activate transcription (4–9). However, imperfect ERE sequences are more commonly found in endogenous, estrogen-responsive genes. Although these imperfect EREs confer estrogen responsiveness to target genes, they are typically less potent activators of transcription than the consensus ERE (10–17).

To determine how various ERE sequences differentially regulate gene expression, we have used the consensus ERE and two imperfect EREs to examine the ER-ERE interaction in detail and determine the effects of this interaction on transcription activation. The two imperfect EREs used in this study, the *X. laevis* vitel-

A

CONS
ERE   ERE2   pS2 ERE

Ab - + - + - +

ER-Ab →

ER →

Free DNA →

B CONS ERE   ERE 2   pS2 ERE

— ERE NS — ERE NS — ERE NS

Fig. 1. Partially Purified Yeast-Expressed hER Interacts Specifically with DNA Fragments Containing Consensus and Imperfect EREs

Partially purified, yeast-expressed hER (285 fmol) was incubated with 427-bp ^{32}P-labeled DNA fragments, each containing either the consensus ERE, the vitellogenin B1 ERE2, or the pS2 ERE near the 3′-end. ER-DNA mixtures were fractionated on a nondenaturing acrylamide gel. The gel was

logenin B1 ERE2 (AGTCAnnnTGACC, Ref. 18) and the human pS2 ERE (GGTCAnnnTGGCC, Ref. 19), differ from the consensus vitellogenin A2 ERE by a single nucleotide in either the 5′- or 3′-half of the palindrome, respectively. We have determined the affinity of ER for the consensus and imperfect ERE sequences, the magnitude of the ER-induced DNA bending in DNA fragments containing either a consensus or an imperfect ERE sequence, and the ability of simplified reporter constructs containing consensus and imperfect EREs to impart estrogen-induced transcription activation.

RESULTS

Partially Purified, Yeast-Expressed Human ER (hER) Binds Specifically to the Consensus ERE, the Vitellogenin B1 ERE2, and the pS2 ERE

Because much of our investigation involves the interaction of ER with ERE-containing DNA fragments in electrophoretic assays, we wanted to be sure that the protein-DNA complexes we observed were ER-specific. ^{32}P-labeled DNA fragments (427 bp) containing either the X. laevis vitellogenin A2 consensus ERE, the X. laevis vitellogenin B1 ERE2, or the human pS2 ERE were incubated with partially purified, yeast-expressed hER. When this protein-DNA mixture was fractionated on a nondenaturing acrylamide gel, one major protein-DNA complex was observed (Fig. 1, panel A, ER→) as well as a minor, more rapidly migrating complex. To determine whether ER binding was responsible for the formation of these complexes, antibody supershift assays were carried out. When the ER-specific monoclonal antibody H222 was included in the binding reaction with the ^{32}P-labeled DNA fragments and the yeast-expressed hER, both the major and the minor protein-DNA complexes were supershifted (Fig. 1, panel A, ER-Ab→). Thus, ER was present in both of the complexes observed with each of the EREs. The presence of the minor complex may reflect ER proteolysis, the presence of a slightly different population of proteins involved in ER interaction with the ERE, or a combination of these events.

To demonstrate that the ER interacted specifically with the ERE present in the large DNA fragments used, competition assays were carried out. The 427-bp ^{32}P-labeled DNA fragments containing either the consen-

dried and subjected to autoradiography. Panel A, The ER-specific antibody H222 was included in the ER-DNA incubations as indicated (Ab +). Free DNA, ER-DNA complexes (ER), and ER-DNA complexes containing H222 (ER-Ab) are indicated to the *left* of the autoradiogram. Panel B, No additional competitor DNA (−) or 45 ng of a 54-bp DNA fragment containing either a single consensus ERE (ERE) or a nonspecific DNA sequence (NS) were included in the binding reaction.
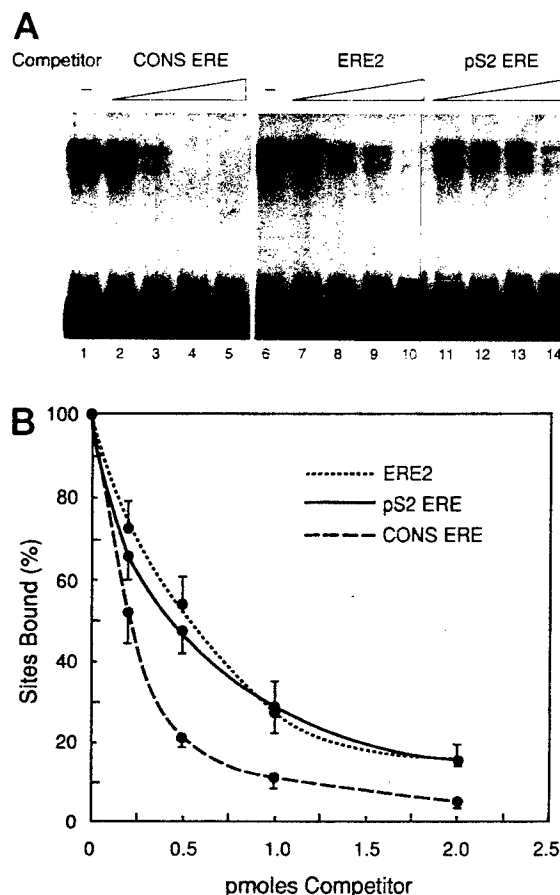
sus ERE, the vitellogenin B1 ERE2, or the pS2 ERE were incubated with the yeast-expressed hER in the absence or in the presence of a 54-bp DNA fragment containing either a nonspecific DNA sequence or the consensus ERE. The DNA fragment containing a consensus ERE, but not the DNA fragment containing the nonspecific DNA sequence, was capable of successfully competing for ER binding (Fig. 1, panel B). Thus, the receptor interacted specifically with each of the ERE-containing DNA fragments used in these studies.

The Affinity of the ER for the Consensus ERE is 2-Fold Higher Than for Either the Vitellogenin B1 ERE2 or the pS2 ERE

The expression of ERE-containing target genes is influenced by the affinity of the ER for the ERE. We noted that although the migration of the ER-DNA complexes in our electrophoretic mobility shift assays was very similar for each of the EREs tested, there was a noticeable difference in the amount of ER-DNA complex formed. More ER-DNA complex was formed with DNA fragments containing the consensus ERE than with DNA fragments containing either the vitellogenin B1 ERE2 or pS2 ERE. To determine whether this difference in complex formation was due to a difference in the affinity of ER for the consensus and imperfect EREs, competition experiments were carried out. The yeast-expressed hER was incubated with the ^{32}P-labeled DNA fragment containing a consensus ERE in the absence and in the presence of increasing amounts of unlabeled annealed oligos containing either the consensus ERE sequence, the vitellogenin B1 ERE2 sequence, or the pS2 ERE sequence. The oligo containing the consensus ERE competed most effectively for ER binding (Fig. 2, panel A). A 50% reduction in ER-DNA complex formation was observed when 0.2 pmol of the unlabeled oligo containing the consensus ERE was included in the binding reaction (panel B). In contrast, nearly 0.5 pmol of the pS2 ERE or the vitellogenin B1 ERE2 oligo was required to successfully reduce binding of ER to the ^{32}P-labeled DNA fragment to 50%. Thus, the affinity of the ER was about 2-fold higher for the consensus ERE than for either of the imperfect EREs.

The Yeast-Expressed hER Bends DNA Fragments Containing the Consensus ERE, the Vitellogenin B1 ERE2, and the pS2 ERE to the Same Extent
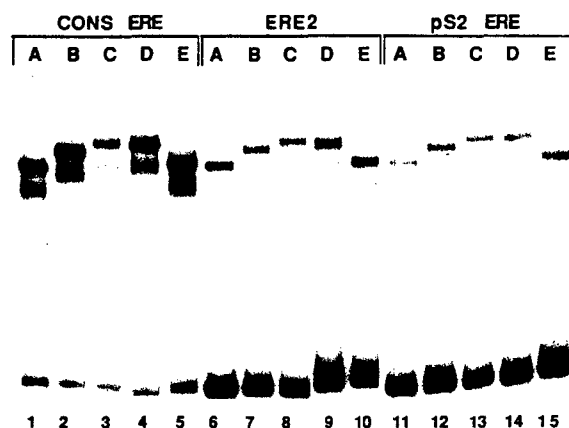
A number of studies have demonstrated that binding of transcription factors, including a number of steroid hormone receptors, induces DNA to bend (20–30). However, all of the studies investigating the role of ER-induced DNA bending thus far have been carried out using the consensus ERE (24, 25, 30). To compare the bending angles induced by ER binding to DNA fragments containing consensus and imperfect EREs, the circular permutation vectors B3ConsERE, B3ERE2, and B3pS2ERE were digested with appropriate restriction enzymes to produce 427-bp DNA

Fig. 2. Partially Purified Yeast-Expressed hER Has Higher Affinity for the Consensus ERE Than for the Vitellogenin B1 ERE2 or the pS2 ERE

Panel A, ^{32}P-labeled DNA fragments each containing a single consensus ERE were incubated in the absence or in the presence of increasing amounts (0, 0.19, 0.48, 0.96, or 1.9 pmol) of unlabeled annealed oligos containing either the consensus ERE, the vitellogenin B1 ERE2. or the pS2 ERE. The ER-DNA complexes were fractionated on an acrylamide gel. The gel was processed as described in Fig. 1. Panel B, The amount of ER-ERE complex was set at 100% and the extent of binding inhibition is indicated. Data from seven competition experiments were combined and values are presented as the mean ± SE.

fragments, each of which contained either the consensus ERE, the vitellogenin B1 ERE2, or the pS2 ERE located at various positions within the DNA fragment. The base pair composition of these large DNA fragments was identical, except for the ERE sequence. After end-labeling and purification, the 427-bp DNA fragments were incubated with yeast-expressed hER and fractionated on a nondenaturing acrylamide gel. The migration patterns of the ER-DNA complexes, regardless of whether the DNA fragments contained a consensus or an imperfect ERE, were very similar. When the ERE was located near the end of the DNA fragment, the ER-DNA complexes migrated more rapidly (Fig. 3, fragments A and E) than when the EREs were at intermediate positions within the DNA frag-

Fig. 3. Partially Purified Yeast-Expressed hER Bends DNA Fragments Containing Consensus and Imperfect EREs
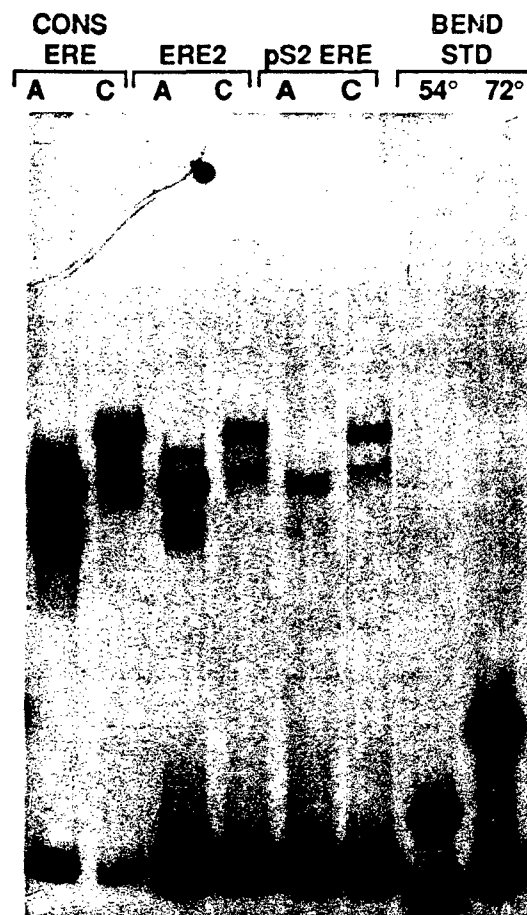
[32]P-Labeled DNA fragments (A-E), each containing a consensus ERE (Cons ERE) or an imperfect ERE (ERE2, pS2 ERE), were incubated with 570 fmol of partially purified yeast-expressed hER and fractionated on an 8% nondenaturing acrylamide gel. The location of the ERE was either at the 5'-end (fragment A), between the 5'-end and the middle (fragment B), in the middle (fragment C), between the 3'-end and the middle (fragment D), or at the 3'-end (fragment E) of the DNA fragment. The gel was processed as described in Fig. 1.

ment (fragments B and D). The most slowly migrating ER-DNA complexes were those that contained an ERE in the middle of the DNA fragment (fragment C). The migration pattern of these ER-DNA complexes indicated that the receptor induced bending in DNA fragments containing consensus and imperfect EREs.

To accurately determine the degree of the ER-induced DNA bending with each of the EREs, DNA bending standards containing 54° and 72° bends were included on the same gels as the ERE-containing DNA fragments and used to quantitate the bending angles. As seen in Fig. 4, the migration of the ER-DNA complexes containing consensus and imperfect EREs was very similar when the EREs were at the end (fragment A) or in the middle (fragment C) of the DNA fragments. By comparing the relative mobility of the DNA-bending standards and the ER-DNA complexes, we determined that the degree of bending induced by ER binding to each set of DNA fragments was nearly identical. The degree of bending induced by binding of ER to DNA fragments containing the consensus ERE, the vitellogenin B1 ERE2, or the pS2 ERE was ~65° using 8–13 independent determinations (Table 1).

ER Present in Chinese Hamster Ovary (CHO)-ER Nuclear Extracts Bends DNA Fragments Containing Consensus and Imperfect EREs to the Same Extent as Partially Purified Yeast-Expressed hER

ER-associated proteins have been implicated in assisting receptor binding to the ERE in *in vitro* gel shift

Fig. 4. Partially Purified Yeast-Expressed hER Induces Similar Bends in DNA Fragments Containing Consensus and Imperfect EREs
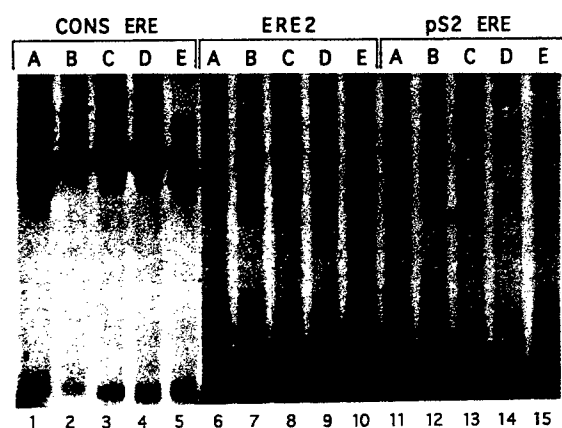
[32]P-Labeled DNA fragments, each containing either a consensus ERE (Cons ERE) or an imperfect ERE (ERE2, pS2 ERE) at the end (A) or in the middle (C) of the DNA fragment, were incubated with 570 fmol of partially purified yeast-expressed hER. The ER-DNA complexes were fractionated on an acrylamide gel. The gel was processed as described in Fig. 1. DNA bending standards containing 54° and 72° intrinsic bends were run on the same gel and used to determine the degree of ER-induced DNA bending.

assays (31–33). Thus, it was possible that the partially purified yeast-expressed hER might not have been associated with the full complement of proteins required for receptor binding in mammalian target cells. Therefore, we used extracts from CHO cells that had been stably transfected with an inducible hER expression vector and can express up to 5 million ER sites per cell (CHO-ER cells) as a comparison (34). Nuclear extracts were prepared from induced CHO-ER cells that had been exposed to 17β-estradiol for 40 min before extract preparation and were used in circular permutation analysis experiments. As seen in Fig. 5, the mobility of the ER-DNA complexes formed with nuclear extracts from CHO-ER cells was similar to the mobility of the ER-DNA complexes formed with partially purified yeast-expressed hER. The degree of

Table 1. Determination of DNA Bending Angles for Consensus and Imperfect EREs

	Angle	n
Consensus ERE	65.9 ± 1.4	8
Vitellogenin B1 ERE2	65.1 ± 0.7	13
pS2 ERE	65.9 ± 0.9	12

Using DNA bending standards containing 54° and 72° intrinsic bends, a plot of relative mobility vs. DNA bending angles was constructed. The bending angles for each of the ER-DNA complexes were determined by interpolation from this plot. Data are presented as the mean ± SE with the number (n) of individual determinations indicated.

Fig. 5. ER Present in CHO-ER Nuclear Extracts Bends DNA Fragments Containing Consensus and Imperfect EREs to the Same Extent as the Partially Purified Yeast-Expressed hER

^{32}P-labeled DNA fragments (A-E), each containing a consensus ERE (Cons ERE) or an imperfect ERE (ERE2, pS2 ERE) were incubated with CHO-ER nuclear extracts containing 20 fmol ER. The ER-DNA complexes were fractionated on an acrylamide gel. The gel was processed as described in Fig. 1.

bending induced by ER binding to DNA fragments containing the consensus ERE, the vitellogenin B1 ERE2, or the pS2 ERE was the same. Thus, the ER present in partially purified yeast extracts and in CHO-ER nuclear extracts binds and bends DNA fragments containing consensus and imperfect EREs in a similar manner.

It should also be noted that the ER present in the CHO-ER extracts is a mutant form with a valine replacing a glycine at amino acid 400 (34, 35). This glycine to valine mutation is associated with decreased affinity of the receptor for estradiol at 25 C (35). However, since we have previously demonstrated that unoccupied and occupied ER present in MCF-7 whole cell extracts bends DNA to the same extent and in the same direction (25, 36) and procedures were carried out at 4 C, except for a 15-min incubation at ambient temperature, one would predict that the mutant receptor would interact with the ERE in a manner similar to the wild type receptor. Indeed, no differ-

ences in the receptor-DNA interaction were detected with these two receptor forms.

The Consensus ERE Is a More Potent Activator of Transcription Than the Vitellogenin B1 ERE2 or the pS2 ERE

Although a number of laboratories have examined the ability of the consensus ERE, the vitellogenin B1 ERE2, and the pS2 ERE to activate transcription using transient cotransfection assays (6, 9, 10, 19), these studies were carried out using different reporter constructs, cell lines, and transfection protocols, making it difficult to compare the ability of each individual ERE to activate transcription. To more directly assess the ability of the consensus and imperfect EREs to activate transcription, we inserted one copy of each ERE into a chloramphenicol acetyltransferase (CAT) reporter plasmid containing only a TATA sequence and then used these vectors in transient cotransfection experiments. The CAT reporter plasmids ConsERE-CAT, ERE2-CAT, and pS2ERE-CAT, each containing one copy of either the consensus ERE, the vitellogenin B1 ERE2, or the pS2 ERE, respectively, were cotransfected along with the hER expression vector pCMV5-hER (37) into CHO cells, and the level of CAT expression was monitored. To correct for variation in transfection efficiency, pCH110, a β-galactosidase expression plasmid that is constituitively expressed, was used as an internal control in all transfections. As seen in Fig. 6, the most potent activator of transcription was the promoter containing a consensus ERE. CAT activity was 12.7-fold greater when cells transfected with ConsERE-CAT were exposed to 10 nM 17β-estradiol than when cells were maintained in a hormone-free environment. A comparison of the imperfect EREs demonstrates that the pS2 ERE (pS2ERE-CAT) was a more powerful transcription activator than the vitellogenin B1 ERE2 (ERE2-CAT). When CHO cells were transfected with ERE2-CAT or pS2ERE-CAT, CAT activity was 2.4-fold and 3.8-fold higher, respectively, when cells were exposed to 17β-estradiol than when cells were maintained in a hormone-free environment. Although the pS2 ERE, which differs from the consensus ERE by a single base pair mismatch in the 3'-half of the palindrome, was a more potent activator of transcription than the vitellogenin B1 ERE2, which differs from the consensus ERE by a single base pair substitution in the 5'-half of the palindrome, these imperfect EREs were significantly less potent activators of transcription than the consensus ERE.

Since we know that ER binding induces a directed bend in ERE-containing DNA fragments (36), we also wanted to determine whether placing the ERE on the opposite side of the DNA helix would affect CAT expression of our reporter plasmids. The EREs in ConsERE-CAT, ERE2-CAT, and pS2ERE-CAT were separated from the TATA sequence by 28 bp or 2.6 helical turns. By inserting an additional 4 bp between the TATA sequence and the ERE in these plasmids,
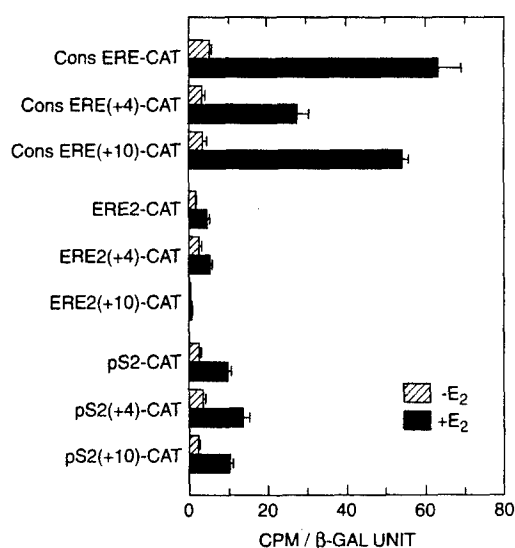
Fig. 6. The Consensus ERE, the Vitellogenin B1 ERE2, and the pS2 ERE Activate Transcription to Different Extents

CHO cells were transfected with CAT reporter plasmid, hER expression plasmid, β-galactosidase expression plasmid, and pTZ nonspecific DNA using the calcium phosphate coprecipitation method as described in *Materials and Methods*. Cells were treated with ethanol vehicle ($-E_2$, *striped bars*) or 10 nM 17β-estradiol ($+E_2$, *solid bars*). Each reporter construct was included in at least six independent transfection experiments. Values are presented as the mean ± SE.

ConsERE(+4)-CAT, ERE2(+4)-CAT, and pS2ERE(+4)-CAT, respectively, were formed, which contained 32 bp or three helical turns between the ERE and the TATA sequence. Cotransfection experiments carried out with these vectors demonstrated that the consensus ERE [ConsERE(+4)-CAT] was again the most potent activator of transcription. The level of CAT activity attained when CHO cells were exposed to 10 nM 17β-estradiol was 9.6-fold greater than when cells were maintained in an estrogen-free environment. However, the level of CAT expression observed when estrogen-treated cells were transfected with ConsERE(+4)-CAT was 55% less than when estrogen-treated cells were transfected with ConsERE-CAT ($P < 0.05$).

In contrast to our findings with the consensus ERE, in which the addition of 4 bp led to a decrease in promoter activity, insertion of 4 bp between the pS2 ERE and the TATA sequence [pS2(+4)-CAT] increased promoter activity by 35% when compared with the parental pS2-CAT vector ($P < 0.05$). The pS2 promoter containing 4 additional base pairs between the ERE and the TATA sequence [pS2(+4)-CAT] was again a more potent activator of transcription than the vitellogenin B1 ERE2, which contained 4 additional base pairs [ERE2(+4)-CAT]. No significant change in transcription activation or hormone responsiveness was observed when 4 additional base pairs were inserted between the vitellogenin B1 ERE2 and the TATA sequence. Thus, by increasing the distance between the ERE and TATA sequence from 2.6 to 3 helical turns, we observed decreased, increased, or

similar levels of transcription with the consensus ERE, pS2 ERE, and the vitellogenin B1 ERE2, respectively.

The transfection experiments seemed to indicate that transcription of our reporter plasmids is dependent upon the orientation of the ERE and TATA sequence. If this hypothesis is correct, we should be able to restore transcription to the levels observed with the parent reporter plasmids by restoring the orientation of the ERE and TATA sequence. Therefore, 10 bp were inserted between the ERE and TATA sequence in the parent vectors ConsERE-CAT, ERE2-CAT, and pS2ERE-CAT to create ConsERE(+10)-CAT, ERE2(+10)-CAT, and pS2ERE(+10)-CAT. The ERE and TATA sequence in these newly synthesized subclones were separated by 38 bp or 3.6 helical turns and were located on opposite sides of the DNA helix. Thus, the orientation of the ERE and TATA sequence in these vectors and in the parent vectors was nearly identical. As predicted, the level of transcription observed was very similar when the consensus ERE and TATA sequence were separated by 2.6 or 3.6 helical turns. Likewise, the level of transcription observed was very similar when the pS2 ERE and TATA sequence were separated by 2.6 or 3.6 helical turns. The decreased transcription observed when cells were transfected with ERE2(+10)-CAT may be due to the increased distance of the relatively weak vitellogenin B1 ERE2 from the TATA sequence. Taken together, these transfection studies demonstrate that consensus and imperfect EREs differentially affect transcription and that the level of transcription observed is dependent upon the orientation of the ERE and TATA sequence.

DISCUSSION

Formation of the ER-ERE Complex

X-ray crystallographic studies have demonstrated that the ER DNA-binding domain binds to the consensus ERE as a homodimer (38). Four amino acid residues present in each DNA-binding domain monomer form hydrogen bonds with nucleotides present in the consensus ERE (38). Two of the amino acids involved in hydrogen bond formation are lysine and glutamic acid, which interact with the first guanine residue present in the 5'-half of the palindrome and the adenine residue present in the 3'-half of the palindrome, respectively, of the consensus ERE (GGTCAnnnTGACC, Ref. 38). Thus, it is not surprising that a change in one of these bases, as is found in the vitellogenin B1 ERE2 (AGTCAnnnTGACC) or the pS2 ERE (GGTCAnnnTGGCC), could have substantial effects on the ER-ERE interaction. In the case of the vitellogenin B1 ERE2, the substitution of an adenine for the guanine results in side chain rearrangement of the lysine residue (39) and, as we have demonstrated in this study, a 2-fold decrease in affinity of ER for the ERE. Although the specific contacts between the ER DNA-binding domain and the pS2 sequence have not been defined by crystal-

lographic analysis, the replacement of a donor-acceptor residue (adenine) with an acceptor residue (guanine) would almost certainly result in rearrangement of the glutamic acid residue, which serves as a hydrogen bond acceptor and requires a donor residue for hydrogen bond formation (40). Such a rearrangement of the amino acid-base pair interaction could explain the decreased affinity of ER for the pS2 ERE relative to the consensus ERE. Our affinity determinations for the imperfect EREs are comparable to, but somewhat lower than, the 3- to 5-fold decreases in affinity previously reported for the vitellogenin B1 ERE2 and the pS2 ERE when compared with the consensus ERE (9, 11, 41). These modest differences in relative affinity are probably due to variations in buffer conditions, concentration of reagents, the source of ER, and the composition of the ERE-containing DNA fragments.

Effects of ER Affinity on Transcription Activation

The reduced affinity of the receptor for the imperfect EREs was reflected in their diminished capacity to activate transcription. The imperfect EREs were significantly weaker activators of transcription than the consensus ERE in all of the reporter constructs we tested. Although the imperfect EREs had similar affinities for ER, the vitellogenin B1 ERE2 was a significantly weaker activator of transcription than the pS2 ERE, indicating that the affinity of ER for an ERE is not the sole determinant involved in transcription activation. It is interesting to note that the vitellogenin B1 ERE2 is not required to function autonomously in the endogenous gene to confer estrogen responsiveness since the X. laevis vitellogenin B1 gene contains two imperfect EREs, ERE1 and ERE2, located in close proximity (18). Although neither ERE1 nor ERE2 is able to significantly activate transcription alone, together they cooperatively activate transcription to the same extent as a single consensus ERE (11). The pS2 gene, in contrast, relies on a single imperfect ERE to confer estrogen responsiveness (19).

Effects of ERE and TATA Sequence Phasing on Transcription Activation

Our transfection studies were designed to assess the ability of consensus and imperfect EREs to activate transcription. To avoid the complicating effects of synergism with upstream elements, we constructed reporter plasmids with a single ERE located a short distance from the TATA sequence. Previous studies have demonstrated that the TATA-binding protein binds to the TATA sequence and bends DNA toward the major groove of the DNA helix (42). Since this is the same orientation as an ER-induced DNA bend (36), we sought to determine whether changes in phasing of the ERE and TATA sequence would elicit changes in transcription of our simplified promoters. Transfection studies demonstrate that the consensus ERE was a

more potent activator of transcription than either of the imperfect EREs tested but that proper placement of the ERE within these simplified promoters was crucial for maximal transcription activation. The highest level of CAT expression was observed when estrogen-induced CHO cells were transfected with a reporter plasmid containing the consensus ERE and TATA sequence on opposite sides of the DNA helix [Fig. 6, ConsERE-CAT, ConsERE(+10)-CAT]. The level of CAT activity was diminished by nearly 55%, however, when the consensus ERE was on the same side of the DNA helix as the TATA sequence [ConsERE(+4)-CAT]. Paradoxically, the pS2 ERE was a more potent activator of transcription when it was on the same side of the DNA helix as the TATA sequence [pS2(+4)ERE-CAT] than when it was on the opposite side of the DNA helix [pS2ERE-CAT, pS2ERE(+10)-CAT]. The level of CAT expression observed when estrogen-treated CHO cells were transfected with reporter plasmids containing the vitellogenin B1 ERE2 [ERE2-CAT, ERE2(+4)-CAT, and ERE2(+10)-CAT] indicate that this ERE is a substantially weaker activator than either the consensus or pS2 ERE.

Although we have demonstrated that the level of transcription activation observed with our simplified promoters is related to the affinity of ER for an ERE, differences in affinity cannot explain how two imperfect EREs, which have similar affinities for ER, activate transcription to different levels or how variation in spacing between the TATA sequence and the ERE can have such disparate effects in promoters containing the consensus and pS2 EREs. A number of studies have indicated that specific contacts between protein and DNA are often accompanied by conformational changes in protein, DNA, or both (43–49). Nuclear magnetic resonance and crystal structure studies of the ER DNA-binding domain demonstrate that upon binding to the ERE, the ER DNA-binding domain undergoes a DNA-induced conformational change that is involved in formation of a dimerization interface (38, 39, 50). DNA-induced conformational changes have also been documented with the glucocorticoid receptor DNA-binding domain (51, 52) as well as a number of other transcription factors (43–49). DNA footprinting experiments with the glucocorticoid receptor DNA-binding domain suggest that the receptor conformation varies when single-base pair substitutions are made in the glucocorticoid response element (53). It is possible that changes in ER conformation brought about by contact of specific amino acids with the various ERE sequences could elicit changes in ER conformation, which would influence ER-protein interactions and lead to changes in transcription activation. We cannot at this point, however, rule out the possibility that the orientation of the ER-induced DNA bend is different for the consensus and imperfect EREs. Changes in orientation of the ER-induced bends could account for differential ER-protein interaction and variations in transcription activity.

ER-Induced Bending of DNA Fragments Containing Consensus and Imperfect EREs

Partially purified, yeast-expressed receptor was initially used to determine that ER binding induces a 65° bend in DNA fragments containing either the consensus or imperfect EREs. These results contrast with studies of thyroid receptor-induced DNA bending, in which thyroid hormone receptor binding induced smaller bends in DNA fragments containing imperfect thyroid response elements than in DNA fragments containing consensus thyroid response elements (26). To be certain that the yeast-expressed hER did not provide anomalous results, we also used ER expressed in mammalian cells, which might contain receptor-associated factors required for ER binding as well as a receptor population that was more appropriately processed. Although there were distinct differences in the proteins present in these extracts, both the partially purified, yeast-expressed hER and the ER present in CHO-ER nuclear extracts formed ER-DNA complexes that migrated very similarly.

Although we observed no differences in the migration of the ER-DNA complexes with DNA fragments containing either consensus or imperfect EREs using ER from two different sources, significantly more of the yeast-expressed hER (285–570 fmol) was used for gel shift analysis than CHO-ER-expressed receptor (20 fmol). We believe that the large excess of yeast-expressed hER required for ER-DNA complex formation may result from 1) the inability of the yeast cells, which express extremely high levels of ER, to carry out all of the necessary posttranslational modifications required for efficient DNA binding, 2) partial denaturation of the receptor during elution from the estradiol affinity column with 5 M urea resulting in a portion of the receptor population that is capable of binding hormone, but incapable of binding DNA, or 3) removal of proteins during purification that enhance or stabilize ER binding to DNA. Although such auxiliary proteins have been described in MCF-7, HeLa, and yeast systems as well as the CHO-ER cells we have used in these studies (31–33), the loss of a stabilizing cofactor seems unlikely since the relative mobility of the ER-DNA complexes, which is an indication of the size and number of proteins involved, was very similar for the partially purified yeast-expressed hER and the ER present in CHO-ER nuclear extracts. In addition, since recovery of ER-associated proteins appears to be favored when the receptor is occupied by estrogen (32) and our ER purification procedure used an estradiol affinity column and elution with estrogen (25), the purification process may have actually favored the isolation of ER with any associated accessory protein(s).

Regulation of Estrogen-Responsive Genes

The regulation of the simplified promoters we have used in these studies does not approximate the level of complexity involved in modulation of endogenous estrogen-responsive genes. The distance between an ERE and the TATA sequence in an endogenous gene is typically greater than the 2.6–3.6 helical turns we have used in our reporter constructs. However, the presence of multiple transcription factor-binding sites in endogenous promoters may provide the framework around which a network of protein-protein contacts could be formed and allow communication between proteins bound to distant sites. Thus, our transfection studies may provide important clues to help define how endogenous estrogen-responsive genes function. First, the affinity of ER for the ERE is an important indicator of the ability of an individual ERE to activate transcription but is not a guaranteed predictor of the transcriptional strength. Second, the location of an ERE within the promoter appears to be an important determinant of the level of gene expression. ER binding is almost certainly influenced by interaction with transcription factors bound to adjacent and/or more distally located regulatory sequences. Third, the magnitude and orientation of DNA bends induced by binding of ER and other transcription factors may be crucial in determining the level of transcription activation achieved. Appropriately oriented DNA bends could facilitate protein-protein interactions and provide the appropriate structural framework around which an active transcription complex is formed.

MATERIALS AND METHODS

Preparation of Vectors

The circularly permuted, ERE-containing Bend III vectors B3ConsERE, B3ERE2, and B3pS2ERE were constructed using the oligos

5'-CTAGATTACAGGTCACAGTGACCTTACTCA-3' and
5'-GATCTGAGTAAGGTCACTGTGACCTGTAAT-3';
5'-CTAGATTACCAGTCACTGTGACCCTACTCA-3' and
5'-GATCTGAGTAGGGTCACAGTGACTGGTAAT-3'; and
5'-CTAGATTACAGGTCACGGTGGCCATACTCA-3' and
5'-GATCTGAGTATGGCCACCGTGACCTGTAAT-3', respectively. The annealed oligos, which contained Xbal and BglII compatible ends, were ligated to the BglII cut pCY7 (54). Smal digestion and Klenow fill-in of the Xbal site was carried out to create two blunt ends. This blunt-ended DNA fragment was ligated and transformed into the Sure (Stratagene, La Jolla, CA) strain of Escherichia coli. After screening and sequencing, the plasmids were purified using cesium chloride density gradient centrifugation.

For transcription activation studies, the consensus ERE, vitellogenin B1 ERE2, and pS2ERE oligos listed above were annealed and ligated to Xbal- and BglII-digested TATA-CAT (16). The ligation products (ConsERE-CAT, ERE2-CAT, and pS2ERE-CAT, respectively) were used to transform E. cells. ConsERE(+4)-CAT, ERE2(+4)-CAT, and pS2ERE(+4)-CAT were constructed by cutting ConsERE-CAT, ERE2-CAT, and pS2ERE-CAT, respectively, with BglII, filling in with Klenow, and ligating the blunt ends. ConsERE(+10)-CAT, ERE2(+10)-CAT, and pS2ERE(+10)-CAT were constructed by cutting ConsERE-CAT, ERE2-CAT, and pS2ERE-CAT, respectively, with BglII and ligating the annealed oligos 5'-GATCGCGCCT-3' and 5'-GATCAGGCGC-3'. The sequence of each plasmid was verified by sequence analysis. All plasmids were purified in two sequential cesium chloride density gradient centrifugation steps.

To calculate the number of base pairs separating the ERE and TATA sequence, the second nucleotide present in the 3-bp spacer between the ERE half-palindromes and the middle of the 8-bp TATA sequence were used (55).

Gel Mobility Shift Assays

The circular permutation vectors, B3ConsERE, B3pS2ERE, and B3ERE2, were digested with EcoR I, HindIII, EcoRV, NheI, or BamHI to produce 427-bp DNA fragments A, B, C, D, and E containing an ERE at the ends (fragments A and E), at intermediate positions (fragments B and D), or in the middle (fragment C) of the DNA fragments. Labeling and purification of the ERE-containing DNA fragments was carried out as previously described (25, 30).

For gel retardation assays with partially purified, yeast-expressed hER, 285–570 fmol of receptor were incubated with 10 µg BSA, 1 µg polydeoxyinosine-polydeoxycytidine acid (poly dI/dC), 15 mM Tris, pH 7.9, 0.2 mM EDTA, 4 mM dithiothreitol, 10% glycerol, and 20 mM KCl for 15 min at 4 C to bind nonspecific proteins. ^{32}P-Labeled DNA fragments (5000 cpm) were then added (20 µl final volume) and incubated at room temperature for 15 min. For gel shift assays with CHO-ER nuclear extracts, 20 fmol of receptor were incubated with 1 µg sheared, sonicated salmon sperm DNA, 1 µg poly dI/dC, 15 mM Tris, pH 7.9, 0.2 mM EDTA, 4 mM dithiothreitol, 10% glycerol, and 40 mM KCl for 15 min at 4 C. After addition of the ^{32}P-labeled DNA, the mixture was incubated at room temperature for 15 min. Protein-DNA mixtures were fractionated on low ionic strength acrylamide gels (56) at 4 C with buffer recirculation. The monoclonal antibody H222 (1.5 µg) was included in the incubation mixture for supershift experiments. Competition assays contained 45 ng of 54-bp DNA fragments containing either an ERE or a nonspecific DNA sequence (Fig. 1, panel B) or the indicated amounts of excess unlabeled annealed oligos containing either the consensus ERE, the vitellogenin B1 ERE2, or the pS2 ERE sequence (Fig. 2). Quantification of the ER-DNA complex and free probe present in individual lanes was carried out using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and Image Quant software. DNA-bending standards (57) were prepared as previously described (25, 30).

Cell Culture and Preparation of ER-Containing Cell Extracts

CHO cells were maintained in a 5% CO_2 atmosphere in DME/F12 supplemented with 5% charcoal dextran-treated (58) calf serum. CHO-ER cells were maintained in phenol red-free DME/F12 containing 10% heat-inactivated, iron-supplemented calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 25 µg/ml gentamycin with 40 µM cadmium chloride and 50 µM zinc sulfate added when media were changed. Nuclear extracts were prepared from CHO-ER cells that had been exposed to 150 µM zinc sulfate for 24 h to induce ER production. Cells were rinsed with HBSS, harvested with HBSS containing 1 mM EDTA, and pelleted at 800 × g. The cells were counted, resuspended in serum-free medium at 10^6 cells/ml, and incubated with 80 nM 17β-estradiol for 40 min at 37 C. Cells were then pelleted and resuspended in 1 ml cold TE (10 mM Tris, pH 8.0, with 1 mM EDTA) and incubated for 3 min at 4 C. The cells were pelleted, resuspended in 50 mM Tris, pH 7.4, 7.5 mM EDTA, and 10% glycerol with protease inhibitors (50 µg/ml leupeptin, 1 µg/ml pepstatin, 5 µg/ml phenylmethylsulfonylfluoride, and 5 µg/ml aprotinin) and homogenized in a Dounce homogenizer using 45 strokes with a B pestle. The nuclei were pelleted for 10 sec at 16,000 × g and resuspended in 50 mM Tris, pH 8.5, 7.5 mM EDTA, 0.5 M KCl, and 10% glycerol with protease inhibitors for 30 min. The nuclear debris was pelleted at 180,000 × g for 20 min at 4 C. The supernatant was aliquoted and stored at −80 C. Partially purified yeast-expressed hER was prepared as previously described (25). Briefly, stably transfected yeast cells, which had been exposed to copper to induce ER expresion, were pelleted and lysed in a Bead Beater apparatus (BioSpec Products, Bartlesville, OK), according to the manufacturer's instructions, in a buffer that contained 200 mM Tris, pH 9.0, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 M urea, 1% dimethylsulfoxide, and protease inhibitors. The lysate was centrifuged at 30,000 × g. The supernatant was adjusted to 0.7 M NaCl and then applied to a 25-ml estradiol-Sepharose column (59). The column was washed successively with loading buffer, loading buffer containing 0.4 M NaCl and 3 M urea, and loading buffer containing 0.1 M NaCl and 5 M urea. Bound ER was eluted with 2 × 10^{-5} M [^{3}H]estradiol (specific activity = 900 dpm/pmol) in a buffer that contained 25 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, and 5 M urea.

CHO Cell Transfections

Transfections were carried out as described (36). Briefly, calcium phosphate crystals formed in the presence of 3 µg CAT reporter plasmid, 0.2 µg of the β-galactosidase vector, pCH110 (Pharmacia, Piscataway NJ), 5 ng of the hER expression plasmid, pCMV5 hER (37), and 4.8 µg pTZ18U were incubated with CHO cells for 16 h followed by a 3-min 20% glycerol shock. Cells were maintained in complete medium containing either 0.1% ethanol or 10 nM 17β-estradiol for 26–28 h. Harvested cells were pelleted and resuspended in 100 µl 250 mM Tris, pH 7.8. After three freeze-thaw cycles, the cell lysate was pelleted, and the protein concentration of the supernatant was determined using the Bio-Rad (Hercules, CA) protein assay with BSA as a standard. The mixed phase CAT assay was performed using 20 or 35 µg protein as previously described (60). β-Galactosidase activity was determined as described (61) and used to normalize the amount of CAT activity in each sample. Determination of variance was followed by two-sample t tests to determine whether statistical differences between groups existed using SAS and Microsoft Excel.
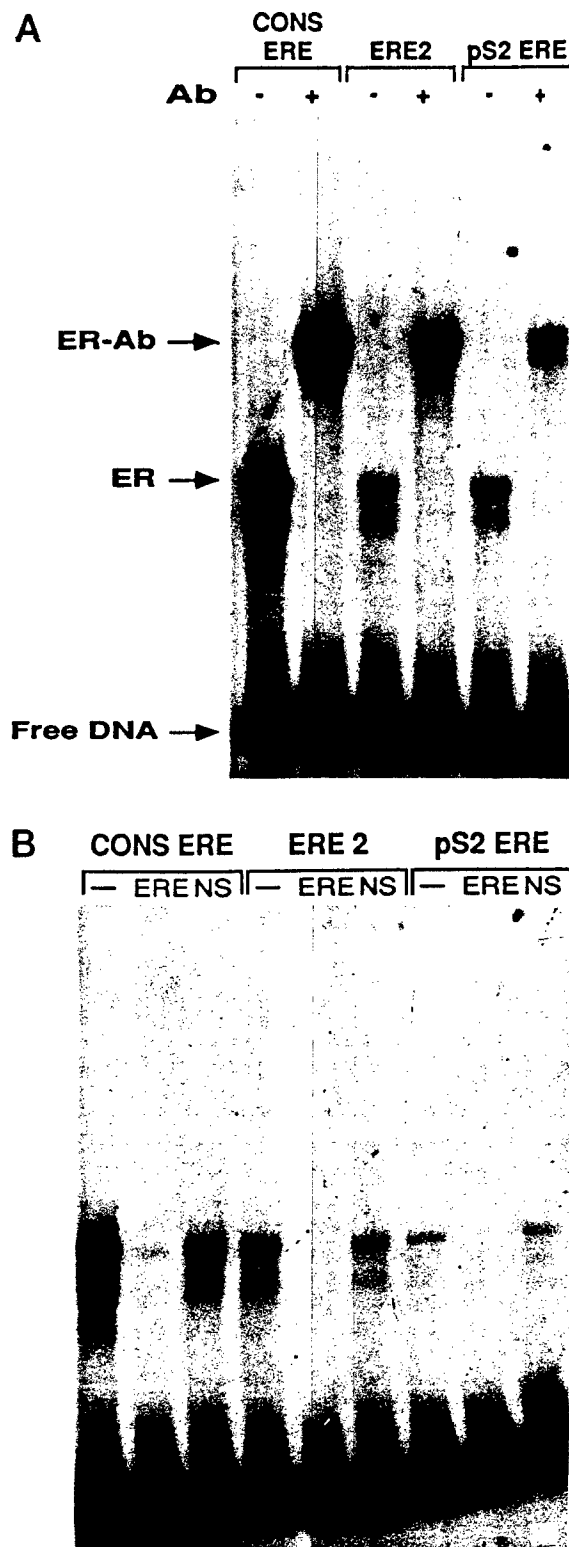
REFERENCES

1. Beato M 1989 Gene regulation by steroid hormones. Cell 56:335–344
2. Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889–895
3. Klein-Hitpass L, Ryffel GU, Heitlinger E, Cato ACB 1988

A 13 bp plaindrome is a functional estrogen responsive element and interacts specifically with estrogen receptor. Nucleic Acids Res 16:647–664

4. Kumar V, Green S, Stack G, Berry M, Jin J-R, Chambon P 1987 Functional domains of the human estrogen receptor. Cell 51:941–951

5. Nigro V, Molinari AM, Armetta I, deFalco A, Abbondanza C, Medici N, Puca GA 1992 Purified estrogen receptor enhances in vitro transcription. Biochem Biophys Res Comm 186:803–810

6. Martinez D, Givel F, Wahli W 1987 The estrogen responsive element as an inducible enhancer: DNA sequence requirements and conversion to a glucocorticoid responsive element. EMBO J 6:3719–3727

7. Klein-Hitpass L, Tsai SY, Greene GL, Clark JH, Tsai M-J, O'Malley BW 1989 Specific binding of estrogen receptor to the estrogen response element. Mol Cell Biol 9:43–49

8. Curtis SW, Korach KS 1990 Uterine estrogen receptor interaction with estrogen-responsive DNA sequences in vitro: effects of ligand binding on receptor-DNA complexes. Mol Endocrinol 4:276–286

9. Ponglikitmongkol M, White JH, Chambon P 1990 Synergistic activation of transcription by the human estrogen receptor bound to tandem responsive elements. EMBO J 9:2221–2231

10. Seiler-Tuyns A, Walker P, Martinez E, Mérillat A-M, Givel F, Wahli W 1986 Identification of estrogen-responsive DNA sequences by transient expression experiments in a human breast cancer cell line. Nucleic Acids Res 14:8755–8770

11. Martinez E, Wahli W 1989 Cooperative binding of estrogen receptor to imperfect estrogen-responsive DNA elements correlates with their synergistic hormone-dependent enhancer activity. EMBO J 8:3781–3791

12. Berry M, Nunez A-M, Chambon P 1989 Estrogen-responsive element of the human pS2 gene is an imperfectly palindromic sequence. Proc Natl Acad Sci USA 86:1218–1222

13. Slater EP, Redeuihl G, Theis K, Suske G, Beato M 1990 The uteroglobin promoter contains a noncanonical estrogen responsive element. Mol Endocrinol 4:604–610

14. Darwish H, Krisinger J, Furlow JD, Smith C, Murdoch FE, DeLuca HF 1991 An estrogen-responsive element mediates the transcriptional regulation of calbindin D-9K gene in rat uterus. J Biol Chem 266:551–558

15. Hyder SM, Stancel GM, Nawaz Z, McDonnell DP, Loose-Mitchell DS 1992 Identification of an estrogen response element in the 3'-flanking region of the murine c-fos protooncogene. J Biol Chem 267:18047–18054

16. Chang TC, Nardulli AM, Lew D, Shapiro DJ 1992 The role of estrogen response elements in expression of the Xenopus laevis vitellogenin B1 gene. Mol Endocrinol 6:346–354

17. Dana SL, Hoener PA, Wheeler DA, Lawrence CB, McDonnell DP 1994 Novel estrogen response elements identified by genetic selection in yeast are differentially responsive to estrogens and antiestrogens in mammalian cells. Mol Endocrinol 8:1193–1207

18. Walker P, Germond J-E, Brown-Luedi M, Givel F, Wahli W 1984 Sequence homologies in the region preceding the transcription initiation site of the liver estrogen-responsive vitellogenin and apo-VLDLII genes. Nucleic Acids Res 12:8611–8626

19. Nunez A-M, Berry M, Imler J-L, Chambon P 1989 The 5' flanking region of the pS2 gene contains a complex enhancer region responsive to oestrogens, epidermal growth factor, a tumour promoter (TPA), the c-Ha-ras oncoprotein and the c-jun protein. EMBO J 8: 823–829

20. van der Vliet PC, Verrijzer CP 1993 Bending of DNA by transcription factors. Bioessays 15:25–32

21. Wolffe AP 1994 Architectural transcription factors. Science 264:1100–1101

22. Nardulli AM, Shapiro DJ 1993 DNA bending by nuclear receptors. Receptor 3:247–255

23. Shapiro DJ, Greene GL, Nardulli AM 1993 Estrogen receptor induced DNA bending. In: VK Moudgil (ed) Steroid Hormone Receptors: Basic and Clinical Aspects. Birkhäuser, Boston, pp 75–105

24. Sabbah M, Le Ricousse S, Redeuilh G, Baulieu EE 1992 Estrogen receptor-induced bending of the Xenopus vitellogenin A2 gene hormone response element. Biochem Biophys Res Commun 185:944–952

25. Nardulli AM, Greene GL, Shapiro DJ 1993 Human estrogen receptor bound to an estrogen response element bends DNA. Mol Endocrinol 7:331–340

26. Leidig F, Shepard AR, Zhang W, Stelter A, Cattini PA, Baxter JD, Eberhardt NL 1992 Thyroid hormone responsiveness in human growth hormone-related genes. J Biol Chem 267:913–921

27. King IN, de Soyza T, Catanzaro DF, Lavin TN 1993 Thyroid hormone receptor-induced bending of specific DNA sequences is modified by an accessory factor. J Biol Chem 268:495–501

28. Lu XP, Eberhardt NL, Pfahl M 1993 DNA bending by retinoid × receptor-containing retinoid and thyroid hormone receptor complexes. Mol Cell Biol 13:6509–6519

29. Prendergast P, Edwards DP, Progesterone receptor induces bending of its target DNA: distinct effects of A, B receptor forms. Program of the 77th Annual Meeting of The Endocrine Society, Washington, DC, 1995, p 55 (Abstract)

30. Nardulli AM, Shapiro DJ 1992 Binding of the estrogen receptor DNA-binding domain to the estrogen response element induces DNA bending. Mol Cell Biol 12:2037–2042

31. Mukherjee R, Chambon P 1990 A single-stranded DNA-binding protein promotes the binding of the purified oestrogen receptor to its responsive element. Nucleic Acids Res 18:5713–5716

32. Halachmi S, Marden E, Martin G, MacKay H, Abbondanza C, Brown M 1994 Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. Science 264:1455–1458

33. Landel CC, Kushner PJ, Greene GL 1994 The interaction of human estrogen receptor with DNA is modulated by receptor-associated proteins. Mol Endocrinol 8:1407–1419

34. Webb P, Lopez GN, Greene GL, Baxter JD, Kushner PJ 1992 The limits of the cellular capacity to mediate an estrogen response. Mol Endocrinol 6:157–167

35. Tora L, Mullick A, Metzger D, Ponglikitmongkol M, Park I, Chambon P 1989 The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties. EMBO J 8:1981–1986

36. Nardulli AM, Grobner C, Cotter D 1995 Estrogen receptor induced DNA bending: orientation of the bend and replacement of an estrogen response element with an intrinsic DNA bending sequence. Mol Endocrinol 9:1064–1076

37. Reese JC, Katzenellenbogen BS 1991 Differential DNA-binding abilities of estrogen receptor occupied with two classes of antiestrogens: studies using human estrogen receptor overexpressed in mammalian cells. Nucleic Acids Res 23:6595–6602

38. Schwabe JWR, Chapman L, Finch JT, Rhodes D 1993 The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. Cell 75:567–578

39. Schwabe JWR, Chapman L, Rhodes D 1995 The oestrogen receptor recognizes an imperfectly palindromic response element through an alternative side-chain conformation. Structure 3:201–213

40. Suzuki M 1994 A framework for the DNA-protein recognition code of the probe helix in transcription factors: the chemical and stereochemical rules. Structure 2:317–326

41. Curtis SW, Korach KS 1991 Uterine estrogen receptor-DNA complexes: effects of different ERE sequences, ligands, and receptor forms. Mol Endocrinol 5:959–966

42. Horikoshi M, Bertuccioli C, Takada R, Wang J, Yamamoto T, Roeder RG 1992 Transcription factor TFIID induces DNA bending upon binding to the TATA element. Proc Natl Acad Sci 89:1060–1064

43. Steitz TA 1990 Structural studies of protein-nucleic acid interaction: the sources of sequence-specific binding. Q Rev Biophys 23:205–280

44. Frankel AD, Kim PS 1991 Modular structure of transcription factors: implications for gene regulation. Cell 65:717–719

45. Pabo CO, Sauer RT 1992 Transcription factors: structural families and principles of DNA recognition. Annu Rev Biochem 61:1053–1095

46. Alber T 1993 How GCN4 binds DNA. Curr Biol 3:182–184

47. von Hippel PH 1994 Protein-DNA recognition: new perspectives and underlying themes. Science 263:769–770

48. Spolar RS, Record MT 1994 Coupling of local folding to site-specific binding of proteins to DNA. Science 263:777–784

49. Erie DA, Yang G, Schultz HC, Bustamante C 1994 DNA bending by Cro protein in specific and nonspecific complexes: implications for protein site recognition and specificity. Science 266:1562–1566

50. Schwabe JWR, Chapman L, Finch JT, Rhodes D, Neuhaus D 1993 DNA recognition by the oestrogen receptor: from solution to the crystal. Structure 1:187–204

51. Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB 1991 Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature 352:497–505

52. Härd T, Kellenbach E, Boelens R, Maler BA, Dahlman K, Freedman LP, Carlstedt-Duke J, Yamamoto KR, Gustafsson J-A, Kaptein R 1990 Solution structure of the glucocorticoid receptor DNA-binding domain. Science 249:157–160

53. LaBaer J, Yamamoto KR 1994 Analysis of the DNA-binding affinity, sequence specificity and context dependence of the glucocorticoid receptor zinc finger region. J Mol Biol 239:664–688

54. Prentki P, Pham M-H, Galas DJ 1987 Plasmid permutation vectors to monitor DNA bending. Nucleic Acids Res 15:10060

55. Kim Y, Geiger JH, Hahn S, Sigler PB 1993 Crystal structure of a yeast TBP/TATA-box complex. Nature 365:512:520

56. Chodosh LA 1989 Mobility shift DNA-binding assay using gel electrophoresis, p.12.2.1–12.2.10. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JD, Smith JA, Struhl K (eds) Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley Interscience, New York

57. Thompson JF, Landy A 1988 Empirical estimation of protein-induced DNA bending angles: applications to λ site-specific recombination complexes. Nucleic Acids Res 16:9687–9705

58. Eckert RL, Katzenellenbogen BS 1982 Effects of estrogens and antiestrogens on estrogen receptor dynamics and the induction of progesterone receptor in MCF-7 human breast cancer cells. Cancer Res 42:139–144

59. Greene G, Nolan C, Engler J, Jensen E 1980 Monoclonal antibodies to human estrogen receptor. Proc Natl Acad Sci USA 77:5115–5119

60. Nielsen DA, Chang T-C, Shapiro DJ 1989 A highly sensitive mixed-phase assay for chloramphenicol acetyl transferase activity in cultured cells. Anal Biochem 179:19–23

61. Herbomel P, Bourachot B, Yaniv M 1984 Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell 39:653–662

Fig. 1. Partially Purified Yeast-Expressed hER Interacts Specifically with DNA Fragments Containing Consensus and Imperfect EREs

Partially purified, yeast-expressed hER (285 fmol) was incubated with 427-bp ^{32}P-labeled DNA fragments, each containing either the consensus ERE, the vitellogenin B1 ERE2, or the pS2 ERE near the 3'-end. ER-DNA mixtures were fractionated on a nondenaturing acrylamide gel. The gel was dried and subjected to autoradiography. Panel A, The ER-specific antibody H222 was included in the ER-DNA incubations as indicated (Ab +). Free DNA, ER-DNA complexes (ER), and ER-DNA complexes containing H222 (ER-Ab) are indicated to the *left* of the autoradiogram. Panel B, No additional competitor DNA (−) or 45 ng of a 54-bp DNA fragment containing either a single consensus ERE (ERE) or a nonspecific DNA sequence (NS) were included in the binding reaction.

logenin B1 ERE2 (AGTCAnnnTGACC, Ref. 18) and the human pS2 ERE (GGTCAnnnTGGCC, Ref. 19), differ from the consensus vitellogenin A2 ERE by a single nucleotide in either the 5'- or 3'-half of the palindrome, respectively. We have determined the affinity of ER for the consensus and imperfect ERE sequences, the magnitude of the ER-induced DNA bending in DNA fragments containing either a consensus or an imperfect ERE sequence, and the ability of simplified reporter constructs containing consensus and imperfect EREs to impart estrogen-induced transcription activation.

RESULTS

Partially Purified, Yeast-Expressed Human ER (hER) Binds Specifically to the Consensus ERE, the Vitellogenin B1 ERE2, and the pS2 ERE

Because much of our investigation involves the interaction of ER with ERE-containing DNA fragments in electrophoretic assays, we wanted to be sure that the protein-DNA complexes we observed were ER-specific. ^{32}P-labeled DNA fragments (427 bp) containing either the *X. laevis* vitellogenin A2 consensus ERE, the *X. laevis* vitellogenin B1 ERE2, or the human pS2 ERE were incubated with partially purified, yeast-expressed hER. When this protein-DNA mixture was fractionated on a nondenaturing acrylamide gel, one major protein-DNA complex was observed (Fig. 1, panel A, ER→) as well as a minor, more rapidly migrating complex. To determine whether ER binding was responsible for the formation of these complexes, antibody supershift assays were carried out. When the ER-specific monoclonal antibody H222 was included in the binding reaction with the ^{32}P-labeled DNA fragments and the yeast-expressed hER, both the major and the minor protein-DNA complexes were supershifted (Fig. 1, panel A, ER-Ab→). Thus, ER was present in both of the complexes observed with each of the EREs. The presence of the minor complex may reflect ER proteolysis, the presence of a slightly different population of proteins involved in ER interaction with the ERE, or a combination of these events.

To demonstrate that the ER interacted specifically with the ERE present in the large DNA fragments used, competition assays were carried out. The 427-bp ^{32}P-labeled DNA fragments containing either the consen-
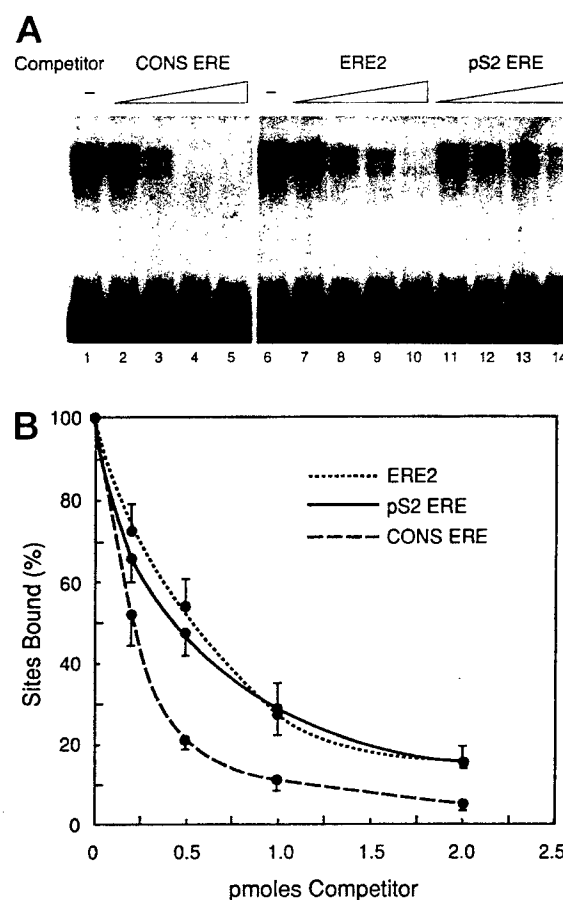
sus ERE, the vitellogenin B1 ERE2, or the pS2 ERE were incubated with the yeast-expressed hER in the absence or in the presence of a 54-bp DNA fragment containing either a nonspecific DNA sequence or the consensus ERE. The DNA fragment containing a consensus ERE, but not the DNA fragment containing the nonspecific DNA sequence, was capable of successfully competing for ER binding (Fig. 1, panel B). Thus, the receptor interacted specifically with each of the ERE-containing DNA fragments used in these studies.

The Affinity of the ER for the Consensus ERE is 2-Fold Higher Than for Either the Vitellogenin B1 ERE2 or the pS2 ERE

The expression of ERE-containing target genes is influenced by the affinity of the ER for the ERE. We noted that although the migration of the ER-DNA complexes in our electrophoretic mobility shift assays was very similar for each of the EREs tested, there was a noticeable difference in the amount of ER-DNA complex formed. More ER-DNA complex was formed with DNA fragments containing the consensus ERE than with DNA fragments containing either the vitellogenin B1 ERE2 or pS2 ERE. To determine whether this difference in complex formation was due to a difference in the affinity of ER for the consensus and imperfect EREs, competition experiments were carried out. The yeast-expressed hER was incubated with the ^{32}P-labeled DNA fragment containing a consensus ERE in the absence and in the presence of increasing amounts of unlabeled annealed oligos containing either the consensus ERE sequence, the vitellogenin B1 ERE2 sequence, or the pS2 ERE sequence. The oligo containing the consensus ERE competed most effectively for ER binding (Fig. 2, panel A). A 50% reduction in ER-DNA complex formation was observed when 0.2 pmol of the unlabeled oligo containing the consensus ERE was included in the binding reaction (panel B). In contrast, nearly 0.5 pmol of the pS2 ERE or the vitellogenin B1 ERE2 oligo was required to successfully reduce binding of ER to the ^{32}P-labeled DNA fragment to 50%. Thus, the affinity of the ER was about 2-fold higher for the consensus ERE than for either of the imperfect EREs.

The Yeast-Expressed hER Bends DNA Fragments Containing the Consensus ERE, the Vitellogenin B1 ERE2, and the pS2 ERE to the Same Extent

A number of studies have demonstrated that binding of transcription factors, including a number of steroid hormone receptors, induces DNA to bend (20–30). However, all of the studies investigating the role of ER-induced DNA bending thus far have been carried out using the consensus ERE (24, 25, 30). To compare the bending angles induced by ER binding to DNA fragments containing consensus and imperfect EREs, the circular permutation vectors B3ConsERE, B3ERE2, and B3pS2ERE were digested with appropriate restriction enzymes to produce 427-bp DNA

Fig. 2. Partially Purified Yeast-Expressed hER Has Higher Affinity for the Consensus ERE Than for the Vitellogenin B1 ERE2 or the pS2 ERE

Panel A, ^{32}P-labeled DNA fragments each containing a single consensus ERE were incubated in the absence or in the presence of increasing amounts (0, 0.19, 0.48, 0.96, or 1.9 pmol) of unlabeled annealed oligos containing either the consensus ERE, the vitellogenin B1 ERE2, or the pS2 ERE. The ER-DNA complexes were fractionated on an acrylamide gel. The gel was processed as described in Fig. 1. Panel B, The amount of ER-ERE complex was set at 100% and the extent of binding inhibition is indicated. Data from seven competition experiments were combined and values are presented as the mean ± SE.

fragments, each of which contained either the consensus ERE, the vitellogenin B1 ERE2, or the pS2 ERE located at various positions within the DNA fragment. The base pair composition of these large DNA fragments was identical, except for the ERE sequence. After end-labeling and purification, the 427-bp DNA fragments were incubated with yeast-expressed hER and fractionated on a nondenaturing acrylamide gel. The migration patterns of the ER-DNA complexes, regardless of whether the DNA fragments contained a consensus or an imperfect ERE, were very similar. When the ERE was located near the end of the DNA fragment, the ER-DNA complexes migrated more rapidly (Fig. 3, fragments A and E) than when the EREs were at intermediate positions within the DNA frag-

Estrogen Receptor Accessory Proteins Augment Receptor-DNA Interaction and DNA Bending

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INTRODUCTION

The estrogen receptor (ER) is a member of the nuclear hormone receptor family of transcription factors. In addition to the steroid receptors, the super-family includes receptors for thyroid hormone and vitamins such as retinoic acid and vitamin D. A number of other orphan nuclear receptors, whose ligands have yet to be identified, have been described as well [1]. For ER, as well as other steroid receptors, binding of ligand results in activation of the receptor, a process that includes conformational changes, posttranslational modifications, and changes in receptor-protein interactions. These changes enable the receptor to bind with high affinity to cis-acting hormone response elements (HREs), typically positioned upstream of hormone-responsive genes. Once bound to these sites, the activated receptor modulates the rate of transcription of responsive genes.

Although much has been learned about the behavior of ER functional domains and the nature of target DNA sequences, the molecular details of ER-mediated transcriptional regulation remain unclear. It is possible that ER enhances the formation of an RNA polymerase II preinitiation complex by stabilizing or recruiting the assembly of a template-committed complex of transcription factors. For progesterone receptor (PR), such a stablized complex is postulated to be poised for rapid initiation of transcription by the polymerase and includes multiple factors other than receptor, such as TFIID, IIA, IIB, and IIE/F [2]. The precise roles of each of these factors in the initiation process are only partially understood. What is clear, however, is that steroid receptors do not act in isolation, but rather in concert with various receptor-associated proteins.

The identities and functions of receptor-associated proteins are only beginning to emerge. Steroid receptors can interact with other transcription activators (eg. AP-1) [3] as well as various co-regulators (eg. CBP and SRC-1) [4-6] and members of the basal transcription apparatus. For example, the basal transcription factor TFIIB interacts with both the progesterone and estrogen

receptors *in vitro* [7]. Furthermore, as measured by an *in vitro* assay, TFIIB was able to stimulate receptor-mediated transcriptional activation, suggesting that interaction of the receptors with TFIIB may be a critical component to receptor-mediated activation. Still other reports suggest that nuclear accessory factors or coactivators are needed for receptor-mediated transactivation. A 55-kDa nuclear accessory factor (NAF) appears to be essential for maximal binding of the vitamin-D receptor to the vitamin-D response element from the human osteocalcin promoter [8]. Similarly, a 65-kDa factor termed triiodothyronine receptor-auxiliary protein (TRAP), which exhibits limited independent DNA binding, enhances thyroid receptor binding to DNA [9]. The non-histone high mobility group chromatin protein, HMG-1, can substitute for an unidentified factor present in partially purified PR fractions and is responsible for promoting PR-DNA binding [10]. More recent studies have identified a protein, Trip-1 (thyroid hormone receptor interacting protein), that interacts with both thyroid hormone receptor (TR) and retinoic-X receptor (RXR) in a ligand-dependent fashion [11]. Trip1 has significant homology with the yeast transcriptional mediator Sug1. Significantly, Trip1 can functionally substitute for Sug1 in yeast, and both proteins interact *in vitro* with the thyroid hormone receptor.

Identification of proteins that associate with activated ER has been the focus of many recent investigations. TIF1 was identified as a protein which stimulated RXR transcriptional activity in yeast and was subsequently shown to potentiate ER activity as well [12]. Another study identified a 45-kDa single-strand DNA-binding protein (DNA-binding stimulatory factor; DBSF) that stimulated the interaction of purified ER with an estrogen response element (ERE) *in vitro* [13]. Biochemical analysis recently revealed a 160-kDa ER-associated protein (ERAP160) that exhibits estradiol-dependent binding to the receptor [14]. Significantly, mutational analysis of the receptor demonstated that its ability to activate transcription paralleled its ability to bind ERAP160. Furthermore, antiestrogens were unable to promote ERAP160 binding and could block the estrogen-dependent association in a dose-dependent manner. In a similar study, another set of ER-associated proteins (receptor-interacting proteins; RIPs) were identified by two *in vitro* techniques,

GST pull-down assay and far-Western blotting [15]. The far-Western technique identified three RIPs with molecular masses of 160, 140 and 80 kDa. The GST pull-down assay failed to detect RIP140 and RIP80, but did detect RIP160 as well as two additional RIPs with molecular weights of 100 and 50 kDa. Importantly, these interactions were only observed with the transcriptionally active, estrogen-occupied ER and were abolished by antiestrogens. It is thought that these proteins may contribute to hormone-dependent transcriptional activation by ER. A recent study suggests that CREB binding protein (CBP) may represent a common, limiting factor that integrates the transcriptional activities of nuclear receptors by interacting with both receptor and SRC-1, p160, and p140 co-activators [4]. In addition, we have previously described four proteins, including hsp70, protein disulfide isomerase (PDI), and two unknown proteins (p48 and p45), that copurify with ER using three chromatographic techniques [16]. Gel shift experiments demonstrated that these ER-associated proteins influenced the ER-ERE interaction [16]. Thus, while a number of receptor-asociated proteins have been identified, the mechanisms by which these proteins alter ER activity *in vivo* is still unknown.

Because many prokaryotic and eukaryotic transcription factors alter DNA structure upon binding to their recognition sequences [17-22], it has been proposed that DNA distortion and bending may be involved in transcription activation. Several members of the nuclear receptor superfamily including estrogen, progesterone, thyroid, retinoid X, and glucocorticoid receptors and the orphan receptor RORα induce conformational changes in DNA structure upon binding to their cognate recognition sequences [23-29]. The TATA binding protein, which is instrumental in forming the basal transcription initiation complex, also induces a sharp bend in DNA [30].

Evidence to support a role for DNA bending in transcription activation includes the observation that intrinsically bent DNA can replace a protein binding site in the promoter and mediate either repression or activation of transcription in a number of systems [31-34]. The ER DNA–binding domain, which is less effective in activating transcription than full length ER, binds specifically to

the ERE and induces a 34° distortion angle in ERE–containing DNA fragments [26]. The full–length human ER, when expressed in yeast, MCF–7, or COS cells, induces a significantly larger 56–65° distortion angle [34, 35]. Thus there appears to be a relationship between the magnitude of DNA bending and transcription activation. Because these earlier experiments with the full–length ER utilized a complex array of cellular proteins in addition to the receptor, it was of interest to examine the ER–ERE interaction using more highly purified ER preparations to determine if ER–associated proteins influence ER–induced DNA distortion and/or bending.

In this study, extracts from CHO-ER cells [36], which express high levels of human ER, were used as a source of affinity purified ER to examine the effects of several associated proteins (hsp70, PDI, p45, p48) on ER-ERE interactions in filter binding and electrophoretic mobility shift assays. Surprisingly, we find that one or more of these proteins influences the absolute ability of purified ER to interact with ERE, but not the rate of association or dissociation of ER and ERE. In addition, the same ER-associated proteins significantly influence the magnitude, but not the direction, of ER-induced bending of ERE-containing DNA fragments. Higher order ER-ERE-protein complexes displayed distorion angles as high as 97°, compared to 62-66° for the smaller and more abundant ER-ERE complexes normally observed. Our results suggest that one or more ER-associated proteins may play an important role in both the DNA binding and bending activities of ER and thus contribute to the overall transcriptional stimulation of target genes.

MATERIALS AND METHODS

Culture of mammalian cells

CHO-ER cells [36] were cultured in Dulbecco's Modified Eagle Medium/Ham F-12 Nutrient Mixture (1:1; Sigma, St. Louis, MO) without phenol red (Sigma) with 10% iron-supplemented newborn calf serum (Sigma) that did not require charcoal treatment, as previously described [16],

44 mM $NaHCO_3$, 1X antibiotic-antimycotic liquid (penicillin, streptomycin, and amphotericin; GibcoBRL, Grand Island, NY), and 5 mg/L insulin. To maintain expression and selection of the ER gene, 50 µM $ZnSO_4$ and 40 µM $CdSO_4$ were also included in the medium.

Cell fractionation

For the preparation of whole cell and nuclear extracts, subconfluent cells were released from tissue culture vessels with a non-enzymatic cell dissociation solution (Sigma, St. Louis, MO). The releasing action was inactivated by the addition of serum-containing media. Cells were pelleted gently at 800 x g for 5 minutes and washed three times with PBS. To prepare whole cell extracts, the cell pellet was resuspended in 4 volumes of ice cold extraction buffer (50 mM Tris, pH 7.9, 2 mM DTT, 400 mM NaCl (high salt buffer), 5 µg/ml aprotinin, 10 µg/ml leupeptin, and 0.2 mM PEFABLOC). Cells were lysed in an ice bath with a dounce (type B pestle) homogenizer, pelleted at 4 C by centrifugation at 10,000 x g, and the supernatant was frozen in aliquots and stored at -80 C. ER content was determined by the controlled-pore glass bead (CPG) assay as previously described [16, 37, 38] following treatment of the extract with excess [6,7-^{3}H]estradiol (Amersham Life Sciences, Arlington Heights, IL).

To obtain nuclear ER, pelleted CHO-ER cells were resuspended in 10 volumes of PBS containing 10% glycerol and 60 nM [6,7-^{3}H]estradiol and incubated for 30 minutes at room temperature with rocking. Cells were pelleted and the procedure was repeated. The final cell pellet was resuspended in four volumes of 50 mM Tris, pH 7.9, 2 mM DTT (salt-free buffer) that contained a protease inhibitor cocktail. The cells were then lysed in an ice bath by dounce homogenization and the mixture was centrifuged for 30 minutes at 10,000 x g at 4 C. The crude nuclear pellet was resuspended in salt-free buffer and centrifuged again to remove residual cytosolic proteins. To extract the retained [6,7-^{3}H]estradiol-ER complex, the crude nuclear pellet was resuspended in four times the original packed cell volume of 50 mM Tris, pH 7.9, 2 mM DTT, 400 mM NaCl

(high salt buffer) containing protease inhibitor cocktail and incubated for 60 minutes on ice with occasional dounce homogenization. The homogenate was centrifuged as before and the supernatant was collected as the nuclear fraction, which was further clarified by centrifugation for 30 minutes at 50,000 x g and stored at -80 C. The [6,7-^{3}H]estradiol-ER content in the nuclear fraction was determined by direct liquid scintillation counting and by specific adsorption to controlled pore glass beads (CPG) as described above.

Purification of hER from CHO-ER extracts

Estradiol-Sepharose Chromatography (ESeph and EATP). To obtain ESeph-purified proteins, 2.5 ml of CHO-ER whole cell extract, adjusted to contain 0.7 M NaCl and 1 M urea, was applied to a 200 μl estradiol-Sepharose column and incubated batchwise for 1 hour at 4 C, as described previously[16]. The column was washed with 20 bed volumes each of loading buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 700 mM NaCl, 1 M urea), and the same buffer with 400 mM NaCl and 3 M urea. Bound ER was eluted with 2 x 10^{-5} M [6,7-^{3}H]estradiol in a buffer that contained 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 200 mM NaCl, and 5 M urea. The yield of ER was determined by specific adsorption to controlled-pore glass beads. To obtain EATP-purified proteins, CHO-ER whole cell extract was treated with ATP prior to purification of hER by E-Seph chromatography, which significantly reduced the amount of associated hsp70, consistent with the reported behavior of hsp70 proteins [39].

DNA-affinity Chromatography (BERE). To obtain BERE-purified proteins, 2.5 ml of CHO-ER whole cell extract was labeled with excess [6,7-^{3}H]estradiol for 1 hour at 4 C and then dialyzed against a buffer containing 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 1 M urea. ER content in the extract was determined by CPG assay[16]. Excess biotinylated ERE was added to the extract at a ratio of 5 pmol of ERE to 1 pmol of ER along with 50 mg poly(dIdC) and 10 mg of the progesterone response element (PRE) (TGACTTGGTTTGGTACAAAATGTT

CTGATCTG) from the MMTV long terminal repeat as carrier DNA. This mixture was incubated for 20 minutes at 22 C, followed by an additional incubation for 40 minutes at 4 C, and applied to a 200 ml UltraAvidin-agarose column and incubated batchwise for 1 hour at 4 C. The column was washed with 20 bed volumes of loading buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 1 M urea). Bound ER was eluted in a buffer containing 20 mM Tris, 1 mM EDTA, 1 mM DTT, 1 M NaCl, 1 M urea and quantitated by CPG assay as well as by direct counting in scintillation cocktail. When CHO-ER nuclear extracts were used, the procedure was the same as described except that incubation with [6,7-^{3}H]estradiol was not necessary since the cells were pre-labeled with [6,7-^{3}H]estradiol in culture.

Labeling of oligonucleotides for filter binding assays

Oligonucleotides (10 pmol) were end-labeled with [γ-^{32}P]-ATP as previously described [16]. Crude radiolabeled oligonucleotides were purified by electrophoresis on 9% polyacrylamide-bis (29:1) gels containing 0.045 M Tris-borate, 0.001 M EDTA, pH 8.0 (0.5X TBE). The desired double-stranded oligonucleotides were located by autoradiography, extracted from excised gel slices, precipitated from ethanol in the presence of 10 μg of tRNA (Sigma, St. Louis, MO) carrier, and pelleted by centrifugation. The resulting pellets were washed with 70% ethanol, dried, and resuspended in 10 mM Tris, pH 8.0, 1.0 mM EDTA [16]. The specific activity of each labeled oligonucleotide was determined prior to purification by thin layer chromatography on polyethyleneimine-impregnated cellulose developed in 0.5 M KH$_2$PO$_4$, pH 3.4. The oligonucleotide remains at the origin, whereas ATP and inorganic phosphate migrate in the direction of the solvent front.

Nitrocellulose filter binding assay

In a 96-well microtiter plate, 30-µl reaction mixtures containing 1 µg poly(dIdC), 30 µg BSA, 20 mM Tris, pH 7.9, 1 mM DTT and 0.1 M NaCl were prepared. 30 fmol of ER were delivered from either a crude extract or a partially purified ER fraction and the plate was incubated on ice for 5 minutes. Specific competitor, when used, was added at this stage at the desired molar excess. The plate was then centriguged briefly in a refrigerated table top centrifuge at 800 rpm to bring all liquid to the bottom of the well. While on ice, 120 fmol of ^{32}P-labeled ERE (37-mer; (AGCTTGTCCAAAGTCAGGTCACAGTGACCTGATCAAA) derived from the vitellogenin A2 gene was added to the side of each well. The plate was again spun briefly to mix the probe with the reaction mixture. The reaction mixture was then incubated for 30 minutes at room temperature. A 96-well nitrocellulose multiscreen plate was prepared in a vacuum manifold (both from Millipore, Bedford, MA) by prewetting each well with 20 mM Tris, pH 7.9, 1 mM DTT, 0.1 M NaCl (washing buffer). 100 µl of washing buffer was left in each well for sample dispersion. Following the 30-minute incubation, samples were transferred from the microtiter plate using a multichannel pipetman into the appropriate wells of the multiscreen plate. Once all samples were transferred, vacuum was applied to the manifold. All wells were then washed three times with 200 µl of washing buffer containing 0.01% NP-40. Once washing was complete, the vacuum was increased to dry the membranes. When dry, the multiscreen plate was removed from the manifold and 40 µl of scintillation fluor was added to each well. The plates were counted in a Packard Top Count microtiter plate scintillation counter.

Preparation of DNA fragments for electrophoretic assays

The circular permutation vector, ERE Bend I [40], was digested with Eco RI, Hind III, Eco RV, Nhe I, or Bam HI to produce 427 bp fragments containing a consensus ERE at the 3' end, an intermediate 3' position, the middle, intermediate 5' position, or at the 5' end of the DNA fragment, respectively. ^{32}P-labeled DNA fragments were prepared as previously described [40].

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All 427 basepair DNA fragments contained the same nucleotide sequence. The only difference in the fragments was the placement of the ERE.

For phasing analysis, the phasing vectors, ERE26, ERE28, ERE30, ERE32, ERE34, and ERE36 [34], each of which contained a consensus ERE separated from an intrinsic DNA bend by 26,28,30,32, 34, or 36 basepairs, respectively, were digested with Eco RI and Hind III. The resulting 281–291 basepair DNA fragments were labeled with [γ^{32}P] ATP as described [34]. DNA bending standards [22] were digested and labeled as previously described [34].

Circular permutation and phasing analysis electrophoretic assays

Gel mobility shift assays were carried out with BERE– ESeph–, and EATP–purified proteins. 250 fmols of BERE–purified proteins or 100 fmols of ESeph– or EATP–purified proteins were incubated with 1 μg poly (dI–dC), 10% glycerol, 8 mM KCl, 15 mM Tris, pH 7.9, 0.2 mM EDTA, ph 8.0, and 4 mM DTT at 4 C for 15 min. The reactions were then incubated at room temperature for 15 min with 10,000 cpm of the ^{32}P–labeled DNA fragment. Protein–DNA mixtures were fractionated on low ionic strength acrylamide gels [41] at 4 C with buffer recirculation. For supershift experiments, 240 ng of the ER–specific monoclonal antibody H222 was included in the binding reaction and the room temperature incubation was extended to 20 min. For competition assays, equimolar amounts of specific or nonspecific competitor were added to the initial binding reaction. 15.3 ng of a 30 bp annealed oligo containing a consensus ERE as the specific competitor. A 54 bp annealed oligo comprised of sequence from the *Xenopus laevis* vitellogenin B1 noncoding sequence was used as the nonspecific competitor.

Calculation of distortion and bending angles

A Molecular Dynamics phosphorimager and Image Quant software (Molecular Dynamics, Sunnyvale, CA) were utilized to determine the migration distance of each ER–DNA complex and free probe. The magnitude of the distortion angle (α_D) was determined by comparing the relative mobility of each ER–DNA complex with the relative mobilities of DNA bending standards [22] as previously described [26, 40]. The magnitude of a directed DNA bending angle (α_B) was determined using the empirical formula of Kerppola and Curran [40, 42]:

$$\tan(k\alpha_B/2) = \frac{A_{PH}/2}{\tan(k\alpha_C/2)}$$

where α_B is the ER–induced distortion angle, α_C is the intrinsic DNA bend angle, A_{PH} is the phasing amplitude, and k is a coefficient used to adjust for electrophoretic conditions. By comparing the relative mobility of 5 sets of DNA bending standards with the known bend angles, a value of k = 0.991 was determined using the formula $\mu_M/\mu_E = \cos(k\alpha_D/2)$, where μ_M is the relative mobility of the ER–DNA complex when the ERE is in the middle of the DNA fragment and μ_E is the relative mobility of the ER–DNA complex when the ERE is at the end of the DNA fragment [22]. To determine if there were statistical differences in distortion and directed bending angles, determination of variance was followed by two–sample t–tests using Microsoft Excel.

RESULTS

Measurement of the rate of ER-ERE association

We first characterized the ER-ERE interaction using a nitrocellulose filter binding assay. The filter binding assay is a simple method of quantitating DNA bound to protein and is based on the ability of nitrocellulose to bind proteins but not double-stranded DNA. Free DNA will pass through the nitrocellulose filter while protein and any interacting DNA is retained. The use of radioactively

labeled DNA allows one to quantitate the association and dissociation rates of the differentially purified ER preparations and to examine the specificity of the ER–ERE interaction. ER and various associated proteins were purified from high salt extracts of CHO-ER cells by specific adsorption of ER to Sepharose-bound estradiol (ESeph, EATP) or biotinylated vitellogenin A2 ERE (BERE), as described previously [16, 43] and summarized in **Table 1**. The association rate of the vitellogenin A2 ERE with each ER mixture was measured by analyzing the DNA binding reaction at time points from 0-60 minutes following the addition of [^{32}P]ERE **(Fig 1)**. As observed previously, the amount of ER-DNA complex formed in the presence of excess unlabeled ERE was significantly greater for the BERE-purified proteins (hsp70/hER/PDI/p48/p45) than for either the ESeph- (hsp70/hER/PDI) or EATP- (hER/PDI) purified proteins. In addition, it appeared that under these experimental conditions, maximal ER-ERE binding occured by 10 minutes for all mixtures. Although the initial rates of association, reflected by the slopes of the curves prior to saturation, appeared to differ for each ER mixture, the large differences in the height of each curve (total binding) made a visual comparison difficult. Reduction of the incubation temperature to 4 C did not alter the profiles significantly (data not shown). Therefore Scatchard analyses as well as analyses of the rates of dissociation of each ER-ERE complex were performed to clarify this issue.

Measurement of the rate of ER-ERE dissociation

A variation of the filter binding assay was used to measure the rate of ER-ERE dissociation. These experiments were performed by incubating CHO-ER nuclear extract or the BERE-, ESeph- or EATP- purified proteins with [^{32}P]ERE. After the reactions had reached equilibrium (30 minutes), they were diluted ten-fold to quench the forward reaction. Subsequent to dilution, an aliquot of each reaction was removed and spotted onto nitrocellulose as the initial time point. Either TE control, ERE, mtERE (2 bp inversion in the second half of the palindrome: -AGGTCAcagTGCACT-), or nonspecific PRE was then added to the reaction mixtures and time

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points were collected for 60 minutes. As shown in **Fig. 2**, no dissociation of ER from [^{32}P]ERE was observed in the absence (TE) or presence of PRE, whereas both mtERE and ERE were able to displace [^{32}P]ERE when present at a 200-fold molar excess. Notably, the dissociation rate profiles among the four ER mixtures were not significantly different within each competitor series (**Fig. 2**). Therefore, the ER-associated proteins do not appear to exert their influence on the rate of ER-ERE dissociation.

Partially purified ER protein complexes exhibit different binding capacities for the vitellogenin A2 ERE

To independently assess any differences in the capacities of ER/ERE interactions, equilibrium saturation binding studies were carried out with the BERE-, ESeph- and EATP-purified proteins. The experiments were performed by incubating fixed amounts (30 fmol) of the BERE-, ESeph- and EATP-purified ER complexes with increasing amounts of [^{32}P]ERE. The equilibrium binding constants were then determined by Scatchard analysis for each partially purified ER-containing fraction. As expected from the association and dissociation rate data, no significant differences in equilbrium dissociation constants (K_d = 3-5 x 10^{-9} M) were observed among these complexes. Only the absolute ER-ERE binding capacities (B_{max}) were different, as shown in **Fig. 3**.

Effect of associated proteins on the ER-ERE interaction in the presence of competitor DNA

The filter binding assay was also used to examine the specificity of the interaction between the ER complexes and the ERE. These experiments were performed by incubating ER complexes with [^{32}P]ERE in the presence of three doses of either ERE, mtERE, or PRE competitor. For each competitor, the binding profiles for the BERE-, ESeph- and EATP-purified ER complexes, as well as ER in nuclear extracts, appeared to be virtually identical (**Fig. 4**), although a difference

between the effectiveness of mtERE and ERE was readily apparent. From these data, we conclude that the specificity of the ER-ERE interaction was not altered by the associated proteins. This result contrasts with previously published gel shift data that appeared to show a greater sensitivity of the ESeph- and EATP-purified proteins to competition by the mtERE than the BERE-purified proteins [16]. However, these earlier experiments were performed under somewhat different binding conditions with only one dose (200-fold molar excess) of competitor. It is also possible that the gel shift assay is more stringent than the filter binding assay and therefore facilitates the disruption of weaker ER-ERE complexes.

ER binds specifically to ERE–containing DNA fragments in gel mobility shift assays.

Although the filter binding assays provided detailed information about the kinetics of ER association and dissociation with the ERE, they did not provide information about the composition of the ER-ERE complex. To determine if the different ER-ERE complexes varied in composition, BERE–, ESeph–, or EATP–purified proteins were incubated with 427 bp ^{32}P-labeled DNA fragments containing a consensus ERE. When the protein–DNA mixtures were fractionated on a low ionic strength, nondenaturing acrylamide gel, five ER–DNA complexes (1,2,3a,3b, and 4) were observed in the BERE–purified proteins and two ER–DNA complexes were observed (1 and 2) with the ESeph– and EATP–purified proteins (**Fig. 5**). Although complexes 3a and 3b were sometimes present in both the ESeph and EATP purified extracts, these complexes were consistently far less prominent than complexes 1 and 2. Antibody supershift experiments were performed to determine if the protein–DNA complexes observed contained ER. When the ER–specific monoclonal antibody H222 was included in the binding reactions, the protein–DNA complexes were supershifted, indicating that ER–specific binding was occurring (**Fig. 5**).

Competition experiments were also carried out to determine if binding of the ER to the ERE–containing DNA fragments was specific. An oligo containing either the consensus ERE or a nonspecific DNA sequence was included in binding reactions. Although the ERE–containing oligo competed with the ^{32}P–labeled probe for ER binding (**Figure 5, Panel B**), the oligo containing a nonspecific DNA sequence failed to compete (**Fig. 5, Panel B, NS lanes**). These data indicated that the 13 bp ERE present in the large ^{32}P–labeled DNA fragment was responsible for the ER–DNA complexes observed.

ER–induced distortion of ERE–containing fragments is influenced by additional proteins

We have previously demonstrated that human ER from transfected COS cell nuclear extracts, MCF–7 whole cell extracts, and partially purified yeast extracts induces 56°–65° distortion angles in ERE– containing DNA fragments [34, 40, 44]. By using the more highly purified ER present in BERE–, ESeph– and EATP–purified mixtures, we were able to determine if the associated proteins (hsp70, PDI, p45 and p48) altered ER–induced distortion of DNA. Each DNA fragment used in these circular permutation assays contained a single consensus ERE located at various positions within the 427 bp fragment. Earlier studies demonstrated that a DNA fragment with a bend in the middle migrates more slowly on an acrylamide gel than a DNA fragment with a bend at the end [22]. Thus, by observing the migration of ER–DNA complexes formed with DNA fragments containing an ERE at the end or in the middle of the DNA fragment, it is possible to detect and quantitate the magnitude of the distortion induced by ER binding to ERE–containing DNA fragments. BERE–, ESeph–, and EATP–purified proteins were incubated with ^{32}P–labeled DNA fragments containing an ERE at the end, at an intermediate position, or in the middle of the fragment. When the ERE was at the 3' or 5' end of the DNA fragment , the migration of the ER–DNA complex was more rapid (**Fig.6, RI and B** respectively) than when the ERE was at an intermediate 3' or 5' position (**H and N** respectively). The ER–DNA complex with the slowest

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migration was formed with the DNA fragments containing an ERE in the middle (**RV**). This differential migration of the ER–DNA complexes indicates that ER binding caused distortion in the DNA fragments. The magnitude of the distortion was calculated by comparing the relative mobility of the ER–DNA complex with the migration DNA bending standards [22]. The results of several combined experiments are shown in **Table 2**. ER–purified proteins induced distortion angles of 62° and 66° in complexes 1 and 2, respectively. These two smaller ER–DNA complexes were observed with all three of the ER mixtures tested. Three higher order complexes were observed in the BERE–purified mixtures, which contain p48 and p45 in addition to the hsp70, ER, and PDI (p55). Complexes 3a and 3b, which were always observed with the BERE–purified proteins, occasionally with the ESeph–purified proteins, but rarely with the EATP–purified proteins, displayed distortion angles of 75° and 93° respectively. The largest distortion angle of 97° was observed only with the BERE–purified proteins (Complex 4). No differences in the center of the bend were detected with any of the ER preparations.

ER–associated proteins influence the magnitude, but not the direction of an ER–induced DNA bend

Phasing analysis was carried out to determine the direction of the DNA bends induced by ER in the BERE–, ESeph–, and EATP–purified mixtures. This method uses DNA fragments that have an intrinsic DNA bend separated from a single consensus ERE by 26, 28, 30, 32, 34, or 36 nucleotides. The spacing between the intrinsic and ER–induced DNA bends is incrementally varied over one turn of the DNA helix so that there will be a point at which the two bends are out of phase and will have the effect of straightening the DNA fragment and a point at which the two bends will be in phase and form a larger overall bend. When the intrinsic and ER–induced DNA bends are in phase, the ER–DNA complex will be inhibited in its migration and when the DNA bends are out of phase, the ER–DNA complex will migrate more rapidly through an acrylamide matrix. By observing the migration of the DNA fragments containing an ERE separated from an

intrinsic DNA bend by various increments, we can determine the direction of an ER–induced DNA bend.

^{32}P–labeled DNA fragments containing an ERE and an intrinsic DNA bend separated by 26–36 basepairs were incubated with BERE–, ESeph–, and EATP–purified proteins and then separated on a nondenaturing acrylamide gel. With all three of the ER mixtures, the DNA fragments that contained 32 basepairs between the ERE and the intrinsic DNA bend migrated most rapidly through the acrylamide gel (**Fig. 7**). This 32 basepair separation places the ERE and the intrinsic DNA bend on the same side of the DNA helix and indicates that the bends are out of phase. Because the intrinsic DNA bend is toward the minor groove of the DNA helix, the ER–induced DNA bend must be directed toward the major groove of the DNA helix. These findings are consistent with previous phasing analysis experiments carried out with MCF–7 and COS expressed ER [34, 35].

Phasing analysis can also be used to determine the degree of directed DNA bending associated with ER binding to ERE–containing DNA fragments. Data from several phasing analysis experiments were combined and utilized to determine the degree of directed ER–induced DNA bending, as previously described[35]. All ER preparations contained complexes 1 and 2, which induced directed DNA bending angles of 5° to 7°. The BERE–purified proteins induced formation of complexes 3a, 3b, and 4, which represented directed DNA bends of 7°, 12°, and 16°.

DISCUSSION

We have used two complementary methods to examine the interaction of BERE–, ESeph– and EATP–purified ER complexes with the vitellogenin A2 ERE [45, 46]. Both filter binding (Figs 1–3) and electrophoretic mobility shift assays (Fig. 5–7) indicate that BERE–purified ER, with its four associated proteins (hsp70, PDI, p48, p45), has a greater capacity for interaction with the

vitellogenin A2 ERE than either ESeph- or EATP-purified ER, in which p48, p45 (ESeph) and hsp70 (EATP) are missing (**Table 1**). These findings are consistent with previously published gel shift experiments [16]. Filter binding analyses were performed to determine whether this differential binding was related to the association (**Fig. 1**) or dissociation (**Fig. 2**) rate of the ER–DNA complex or whether the absolute capacity of the ER and its associated proteins to bind to the ERE differed (**Fig. 3**). Analysis of the rates of association and dissociation for all three ER mixtures revealed no significant difference in these parameters, suggesting that the enhanced formation of the ER–DNA complex with BERE–purified proteins reflected the overall ability of ER and its associated proteins to bind to the ERE. Scatchard analysis demonstrated that the equilibrium ER-ERE dissociation constants ($K_d = 3\text{-}5 \times 10^{-9}$ M) for BERE- ESeph– or EATP–purified proteins were not significantly different (**Fig. 3**). Furthermore, the Scatchard analysis clearly identified an enhanced capacity of the BERE–purified ER mixture to interact with an ERE when compared to the ESeph– and EATP–purified proteins. These data suggest that one or more receptor–associated proteins may facilitate the conversion of ER from an inactive state (unable to bind ERE) to an active state (able to bind to the ERE), or perhaps stabilize the active state, independent from ligand binding activity.

The decreased binding of more highly purified receptors to their cognate recognition sequences has been reported by others [10, 13]. We have observed an inverse relationship between the number of ER–associated proteins present in the ER preparation and the ability of the receptor to interact with ERE. The most highly purified ER preparation, which contains only ER and PDI (EATP; Table 1), was the least able to form stable ER–DNA complexes. The presence of hsp 70 (ESeph–purified proteins) increased ER–DNA complex formation. BERE–purified ER, which contains four detectable associated proteins (**Table 1**), afforded the most ER–DNA complex in the presence of excess ERE. Thus, similar to the DNA–binding stimulatory factor described by Mukherjee [13], ER–associated proteins, and especially p45 and p48, may promote absolute ER DNA–binding activity. Although we cannot rule out the possibility that denaturation contributes to

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the decreased ER activity of highly purified ER, our data strongly suggest that one or more of the proteins we have isolated contributes directly to the formation of ER complexes with enhanced affinity for ERE and increased bending. Our own previously published reconstitution experiments have confirmed that addition of p48/p45 and hsp70 to the EATP-purified ER can enhance the ER-ERE interaction [16]. Significantly, this process could not be mimicked by the addition of other proteins (e.g. albumin, insulin). As shown here and discussed below, these proteins appear to participate in the formation of higher order ER complexes with improved ERE binding and bending abilities, suggesting that these proteins do not simply renature defective ER molecules.

Although the DNA fragments used in circular permutation and phasing analysis experiments were different, the ER–DNA complexes observed were quite similar for both assays. While all three of the ER preparations (**Table 1**) formed complexes 1 and 2, only the BERE–purified proteins consistently formed complexes 3a and 3b, and only BERE–purified proteins formed complex 4 (**Figs. 5–7**). Thus, p45 and p48, which are present in the BERE preparations, but not in the ESeph or EATP preparations, may be instrumental in the consistent formation of complexes 3a and 3b and are absolutely required for the formation of complex 4. Complexes 3a and 3b are sometimes present in small amounts with ESeph–purified proteins, but are rarely observed with EATP–purified proteins. These findings suggest that hsp70, which is present in the ESeph preparation, but not in the EATP preparation, may be involved in the formation of complexes 3a and 3b, but that p45 and p48 are required to maintain these two higher order complexes. We have also observed higher order ER-ERE complexes with MCF–7 whole cell, nuclear, and cytosolic extracts [34], indicating that similar complexes can form *in vivo*. Thus, both circular permutation and phasing analysis experiments indicate that the observed multiplicity of ER–DNA complexes reflects the population of associated proteins present in the different ER preparations. ER and PDI are involved in formation of complexes 1 and 2. Although hsp 70 may be involved in forming Complex 3a and 3b, maintenance of complexes 3a, 3b, and 4 requires the presence of p45 and p48. Interestingly, complexes 1 and 2 have the same mobility as two ER–DNA complexes

formed with yeast–expressed ER, which was purified on an estradiol–sepharose column [44], suggesting that the ER may associate with similar proteins even though the cellular context is distinctly different. The number of ER–DNA complexes described here differs from an earlier study that used the same ER preparations, but a much smaller DNA probe, different gels and buffers, and different receptor:probe ratios [16]. However, the ability of the BERE–purified proteins to more readily form higher order complexes was observed in both studies.

Electrophoretic assays have been used to examine various characteristics of DNA structure. Circular permutation is typically used to detect regions of undirected, increased flexibility in DNA structure and phasing analysis is used to detect bends with a fixed spatial orientation [21]. We have used circular permutation analysis to determine whether ER–associated proteins might alter the magnitude of distortion induced by the binding of ER to ERE–containing DNA fragments. Complexes 1 and 2 induced distortion angles of 62° and 66°, respectively, in ERE–containing DNA fragments with all of the ER preparations utilized. These finding are in agreement with our previous determinations of the distortion angle induced by human ER isolated from yeast, MCF–7, and COS cells [34, 40, 44]. Complexes 3a, 3b, and 4, which were most prominent when BERE–purified proteins were used, induced significantly larger distortion angles of 75°, 93°, and 97°, respectively (Table 2). Thus, receptor–associated proteins were responsible for producing new, higher order ER–DNA complexes, which caused greater distortion in DNA structure. The ER–associated proteins did not, however, appear to alter the distortion angles of complexes 1 and 2.

Phasing analysis was utilized to examine the ability of ER–associated proteins to affect the magnitude and the direction of the ER–induced DNA bending. As seen with the circular permutation experiments, the formation of higher order ER–DNA complexes caused an increase in the magnitude of the directed DNA bend (Table 2). However, the direction of the ER–induced DNA bend, which was toward the major groove of the DNA helix, was unaltered by the presence of the ER–associated proteins. Thus, both circular permutation and phasing analysis experiments

support the idea that ER and its associated proteins promote the formation of higher order complexes (3a, 3b, and 4) that induce greater distortion and directed bending angles in ERE–containing DNA fragments. The ER–induced DNA bend was directed toward the major groove of the DNA helix. This is the same orientation as ROR-, RXR-, and PR-induced bends [23, 24, 47]. The observation that all nuclear receptor superfamily members examined to date induce DNA bends toward the major groove of the DNA helix may result from the homologous structure of these proteins.

The relationship between alterations in DNA structure and transcription activation is unclear. Because such a large number of transcription factors, including nuclear receptors, induce DNA to bend, it has been hypothesized that distortion or bending of DNA might facilitate the interaction of regulatory proteins with members of the basal transcription complex, and thus be required for transcription activation [7, 23, 48]. ER-mediated transactivation probably requires a large repertoire of proteins to maintain function. Association of ER with one set of proteins may maintain the receptor in a quiescent state. The change in ER conformation induced by hormone binding may dissociate some of these proteins and recruit others. Likewise, interaction of ER with DNA, which induces conformational changes in the dimerization interface of the DNA binding domain [49], could initiate more global changes in ER structure and modulate receptor–protein associations. Therefore, we propose a model (Fig. 8) in which the unliganded ER is associated with PDI and hsp70, as well as hsp90, hsp56 and perhaps other as yet unidentified factors. Following ligand binding, hsp90 and hsp56 dissociate, while hsp70 and PDI remain associated with the "activated" ER, although the hsp70 interaction is perhaps weakened[39]. The "activated" ER complex then recruits or stabilizes the binding of at least two additional proteins, p45 and p48, when ER binds to an ERE, resulting in an increased ability of ER to bind ERE. The resulting change in DNA structure generated by the binding of this complex is likely to contribute to effective transcriptional stimulation. In this model, the ER that does not interact with the ERE dissociates from hsp70, thereby rendering it inactive. Additional proteins identified by other

laboratories (e.g. TFIIB, p140, CBP/p300, SRC-1) may participate in one or more of these steps as well [4, 5, 7, 15]. Clearly, a better understanding of the role of p45 and/or p48 in ER action will require the separation and identification of these two proteins. In addition, the contribution of DNA bending to the formation and/or stabilization of an active transcription complex will ultimately be determined by a more detailed structural analysis of a functional transcription unit.

ACKNOWLEDGMENTS

REFERENCES

1.   Parker M. G. Mechanisms of action of steroid receptors in the regulation of gene transcription. *J. Reprod. Fert.* **88** (1990) 717-720.

2.   Klein-Hitpass L., Tsai S., Weigel N., Allan G., Riley D., Rodriguez R., Schraeder W., Tsai M. and O'Malley B. The Progesterone receptor stimulates cell-free transcription by enhancing the formation of a stable preinitiation complex. *Cell* **60** (1990) 247-257.

3.   Pfahl M. Nuclear receptor/AP-1 interaction. *Endocr. Rev.* **14** (1993) 651-658.

4.   Kamei Y., Xu L., Heinzel T., Torchia J., Kurokawa R., Gloss B., Lin S.-C., Heyman R. A., Rose D. W., Glass C. K. and Rosenfeld M. G. A CBP Integrator Complex Mediates

Transcriptional Activation and AP-1 Inhibition by Nuclear Receptors. *Cell* **85** (1996) 403-414.

5. Onate S. A., Tsai S. Y., Tsai M. J. and O'Malley B. W. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270** (1995) 1354-1357.

6. Horwitz K., Jackson T., Bain D., Richer J., Takimoto G. and Tung L. Nuclear Receptor Coactivators and Corepressors. *Mol. Endocrinol.* **10** (1996) 1167-1177.

7. Ing N., Beekman J., Tsai S., Tsai M.-J. and O'Malley B. Members of the steroid hormone receptor superfamily interact with TFIIB (S300-II). *J. Biol. Chem.* **267** (1992) 17617-17623.

8. Sone T., Ozono K. and Pike W. J. A 55-kilodalton accessory factor facilitates vitamin D receptor DNA binding. *Mol. Endocrinol.* **5** (1991) 1578-1586.

9. Darling D., Beebe J., Burnside J., Winslow E. and Chin W. 3,5,3'-triiodothyronine (T_3) receptor-auxiliary protein (TRAP) binds DNA and forms heterodimers with the T_3 receptor. *Mol. Endocrinol.* **5** (1991) 73-93.

10. Prendergast P., Onate S., Christensen K. and Edwards D. Nuclear accessory factors enhance the binding of progesterone receptor to specific target DNA. *J. Steroid Biochem. Molec. Biol.* **48** (1994) 1-13.

11. Lee J. W., Ryan F., Swaffield J. C., Johnston S. A. and Moore D. D. Interaction of thyroid-hormone receptor with a conserved transcriptional mediator. *Nature* **374** (1995) 91-3.

12. Le Douarin B., Zechel C., Garnier J.-M., Lutz Y., Tora L., Pierrat B., Heery D., Gronemeyer H., Chambon P. and Losson R. The N-terminal part of TIF1, a putative

mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic proteins T18. *EMBO J.* **14** (1995) 2020-2033.

13. Mukherjee R. and Chambon P. A single-stranded DNA-binding protein promotes the binding of the purified oestrogen receptor to its responsive element. *Nucl. Acids Res.* **18** (1990) 5713-5716.

14. Halachmi S., Marden E., Martin G., MacKay H., Abbondanza C. and Brown M. Estrogen receptor-associated proteins: Possible mediators of hormone-induced transcription. *Science* **264** (1994) 1455-1458.

15. Cavailles V., Dauvois S., Danielian P. S. and Parker M. G. Interaction of proteins with transcriptionally active estrogen receptors. *Proc. Natl. Acad. Sci. USA* **91** (1994) 10009-10013.

16. Landel C., Kushner P. and Greene G. The interaction of the human estrogen receptor with DNA is modulated by receptor-associated proteins. *Mol. Endocrinol.* **8** (1994) 1407-1419.

17. Gartenberg M. R. and Crothers D. M. DNA sequence determinants of CAP-induced bending and protein binding affinity. *Nature* **333** (1988) 824-831.

18. Shuey D. J. and Parker C. S. Bending of promoter DNA on binding of heat shock transcription factor. *Nature* **323** (1986) 509-513.

19. Verrijzer C. P., van Oosterhout J. A. W. M., van Weperen W. W. and van der Vliet P. C. Pou proteins bend DNA via the Pou-specific domain. *EMBO J.* **10** (1991) 3007-3014.

20. Vignais M. L. and Sentenac A. Asymmetric DNA bending induced by the yeast multifunctional factor TUF. *J. Biol. Chem.* **264** (1989) 8463-8466.

21. Kerppola T. and Curran T. Fos-Jun heterodimers and Jun homodimers bend DNA inopposite orientations: Implications for transcription factor cooperativity. *Cell* **66** (1991) 317-326.

22. Thompson J. and Landy A. Empirical estimation of protein-induced DNA bending angles: Applications to λ site-specific recombination complexes. bending angles: Applications to site-specific recombination complexes. *Nuclei Acids Research* **16** (1988) 9687-9705.

23. McBroom L., Flock G. and Giguere V. The nonconserved hinge region and distinct amino-terminal domains of the RORα orphan nuclear receptor isoforms are required for proper DNA bending and RORα-DNA interactions. *Mol. Cell. Biol.* **15** (1995) 796-808.

24. Lu S., Eberhardt N. and Pfahl M. DNA bending by retinoid X receptor-containing retinoid and thyroid hormone receptor complexes. *Mol. Cell. Biol.* **13** (1993) 6509-6519.

25. Sabbah M. Estrogen receptor-induced bending of the Xenopus vitellogenin A2 Gene hormone response element. *Biochem. Biophys. Res. Comm.* **185** (1992) 944-952.

26. Nardulli A. M. and Shapiro D. J. Binding of the estrogen receptor DNA binding domain to the estrogen response element induces DNA bending. *Mol. Cell. Biol.* **12** (1992) 2037-2042.

27. Petz L., Nardulli A., Kim J., Horwitz K., Freedman L. and Shapiro D. DNA bending is induced by binding of the glucocorticoid receptor DNA binding domain and progesterone receptor to their response element. *J. Steroid Biochem. Molec. Biol.* **In Press** (1996) .

28. Leidig F., Apriletti J., Baxter J. and Eberhardt N. Thyroid hormone receptors induce DNA bending: potential importance for receptor action (Abstr.). *Clin. Res.* **38** (1990) 474A.

29. King I. Thyroid hormone receptor-induced bending of specific DNA sequences is modified by an accessory factor. *Journal of Biological Chemistry* **268** (1993) 495-501.

30. Kim Y., Geiger J., Hahn S. and Sigler P. Crystal structure of a yeast TBP/TATA-box complex. *Nature* **365** (1993) 512-520.

31. Perez-Martin J. and Espinosa M. Protein-induced bending as a transcriptional switch. *Science* **260** (1993) 805-807.

32. Rojo F. and Salas M. A DNA curvature can substitute phage φ29 regulatory protein p4 when acting as a transcriptional repressor. *The EMBO Journal* **10** (1991) 3438.

33. Bracco L. and et.al. Synthetic curved DNA sequences can act as transcriptional activators in Escherichia coli. *The EMBO Journal* **8** (1989) 4289-4296.

34. Nardulli A. M., Grobner C. and Cotter D. Estrogen receptor-induced DNA bending: Orientation of the bend and replacement of an estrogen response element with an intrinsic DNA bending sequence. *Mol. Endocrinol.* **9** (1995) 1064-1076.

35. Potthoff S., Romine L. and Nardulli A. Effects of Wild Type and Mutant Estrogen Receptors on DNA Flexibility, DNA Bending, and Transcription Activation. *Mol Endocrinol* **10** (1996) 1095-1106.

36. Kushner P., Hort E., Shine J., Baxter J. and Greene G. Construction of cell lines that express high levels of the human estrogen receptor and are killed by estrogens. *Mol. Endocrinol.* **4** (1990) 1465-1473.

37. Gormley G., Hospelhorn V., Khan M. and Jensen E. A controlled pore glass bead assay for the measurement of cytoplasmic and nuclear glucocorticoid receptors. *J. Steroid Biochem. Molec. Biol.* **22** (1985) 693-698.

38. Greene G., Harris K., Bova R., Kinders R., Moore B. and Nolan C. Purification of T47D human progesterone receptor and immunochemical characterization with monoclonal antibodies. *Mol. Endocrinol.* **2** (1988) 714-726.

39.   Kost S., Smith D., Sullivan W., Welch W. J. and Toft D. Binding of heat shock proteins to the avian progesterone receptor. *Mol. Cell. Biol.* **9** (1989) 3829-3838.

40.   Nardulli A., Geoffrey Greene, David Shapiro. Human estrogen receptor bound to an estrogen respose element bends DNA. *Mol. Endocrinol.* **7** (1993) 331-340.

41.   Chodosh L., Mobility Shift DNA-binding assay using gel electrophoresis. In *Current Protocols in Molecular Biology* ed F. Ausubel. Greene Publishing Associates and Wiley Interscience, New York (1989), pp. 12.2.1-12.2.10.

42.   Kerppola T. and Curran T. DNA bending by Fos and Jun: The flexible hinge model. *Science* **254** (1991) 1210-1214.

43.   Landel C., Kushner P. and Greene G. Estrogen Receptor Accessory Proteins: Effects on Receptor-DNA Interactions. *Environmental Health Perspectives* **103** (1995) 23-28.

44.   Nardulli A., Romine L., Carpo C., Greene G. and Rainish B. Estrogen Receptor Affinity and Location of Consensus and Imperfect Estrogen Response Elements Influence Transcription Activation of Simplified Promoters. *Mol. Endocrinol.* **10** (1996) 694-704.

45.   Klein-Hitpass L., Tsai S., Greene G., Clark J., Tsai M. and OMalley B. Specific binding of estrogen receptor to the estrogen response element. *Mol. Cell. Biol.* **9** (1989) 43-49.

46.   Klein-Hitpass L., Ryffel G. U., Heitlinger E. and Cato A. C. B. A 13 bp palindrome is a functional estrogen responsive element and interacts specifically with estrogen receptor. *Nucl. Acids Res.* **16** (1988) 647-663.

47.   Prendergast P., Pan Z. and Edwards D. Progesterone receptor-induced bending of its target DNA: Distinct effects of the A and B receptor forms. *Mol. Endocrinol.* **10** (1996) 393-407.

48.   Jacq X., Brou C., Lutz Y., Davidson I., Chambon P. and Tora L. Human TAFII30 is present in a distinct TFIID complex and is required for transcriptional activation by the estrogen receptor. *Cell* **79** (1994) 107-117.

49.   Schwabe J., Neuhaus D. and Rhodes D. Solution structure of the DNA-binding domain of the oestrogen receptor. *Nature* **348** (1990) 458-461.

Source of ER*	Proteins Present (kDa)#	Relative DNA Binding
NE	total nuclear proteins	++++
BERE	70, 66, 55, 48, 45	++++
ESeph	70, 66, 55	++
EATP	66, 55	+

Table 1. Summary and properties of ER-associated proteins isolated by different chromatographic techniques.

*Methods used to isolate ER from CHO-ER cells: Nuclear extract (NE); Site-specific DNA-affinity chromatography (BERE); Estradiol-Sepharose affinity chromatography (ESeph); Estradiol-Sepharose affinity chromatography in the presence of ATP (EATP).

#The identity of the proteins indicated in the table are: 70 = hsp70; 66 = ER; 55 = PDI; 48 and 45 are unidentified. Adapted from Landel, Kushner and Greene [43].

Purified ER	Complex	Distortion Angle	Bend Angle
BERE	1	62 ± 0.9 (5)	6.7 ± 0.1 (3)
	2	66 ± 0.6 (5)	7.0 ± 0.3 (3)
	3a	75 ± 1.9 (5)	6.5 ± 0.2 (3)
	3b	93 ± 1.6 (5)	12.2 ± 2.2 (3)
	4	97 ± 2.5 (5)	15.7 ± 2.3 (3)
ESeph	1	62 ± 0.9 (4)	5.7 ± 0.3 (4)
	2	65 ± 0.4 (4)	6.9 ± 0.3 (5)
EATP	1	62 ± 1.0 (5)	5.4 ± 0.6 (5)
	2	64 ± 0.5 (4)	6.0 ± 0.2 (5)

Table 2. ER–induced distortion and bending angles. Distortion angles and directed bending angles induced by ER binding to ERE–containing DNA fragments were determined by circular permutation and phasing analysis, respectively. Values are reported as the mean + S.E. The number of individual determinations is indicated in parenthesis. The protein composition of each purified ER mixture is listed in **Table 1**.

FIGURE LEGENDS

Figure 1. Graph of the ER-ERE rate of association. ^{32}P-labeled vitellogenin A2 ERE (37-mer; 120 fmol; -AGGTCAcagTGACCT-) was added to partially purified ER complexes (30 fmol) and incubated at 25° C for 0-30 minutes. At the indicated time points, aliquots of the reaction mixture were removed. The samples were immediately applied to nitrocelluose filters and washed thoroughly to stop the reacation. The time course was performed with CHO–ER nuclear extract and BERE–, ESeph–, and EATP–purified proteins.

Figure 2. Graph of the ER-ERE rate of dissociation. Partially purified ER complexes (30 fmol) were incubated for 30 minutes at 25° C with [32P]ERE (120 fmol) to allow the ER-ERE complexes to reach equilibrium. The reaction mixtures were then diluted 10-fold to quench the forward reaction. At indicated time points following the addition of either TE (control) or a 200-fold molar excess of ERE, mtERE (-AGGTCAcagTGCACT-) or PRE, samples were applied to nitrocellulose filters and washed thoroughly. The time course was performed with CHO–ER nuclear extract and BERE–, ESeph–, and EATP–purified proteins.

Figure 3. Scatchard analysis of the [32P]ERE binding affinity and capacity of three different ER complexes. ER complexes (30 fmol) were incubated for 30 minutes at 25° C with five different concentrations of [32P]ERE (30-500 fmol). Each reaction was then applied directly to nitrocellulose filters and washed to stop the reaction and remove unbound [32P]ERE. The negative reciprocal of the slope of each line was used to calculate the equilibrium dissociation constant (K_d). The analysis was performed with BERE–, ESeph–, and EATP–purified proteins.

Figure 4. Graph to assess the specificity of the ER-ERE interaction. Partially purified ER complexes (30 fmol) were incubated for 30 minutes at 25° C with [32P]ERE (120

fmol) in the presence of three different doses of either ERE, mtERE or PRE. The samples were then applied to nitrocellulose filters and washed thoroughly. The analysis was performed with CHO–ER nuclear extract and BERE–, ESeph–, and EATP–purified proteins.

Figure 5. BERE–, ESeph–, or EATP–purified proteins interact specifically with ERE–containing DNA fragments. Nhe I cut ^{32}P–labeled DNA fragments were incubated with BERE–, ESeph–, or EATP–purified proteins and then fractionated on a nondenaturing 8% acrylamide gel. The ER–specific monoclonal antibody H222 was included in the binding reaction as indicated (+H222). Binding reactions contained either no competitor DNA (–), a 100–fold excess of unlabeled ERE (ERE), or a 100–fold excess of unlabeled nonspecific DNA fragment (NS). ER–DNA complexes are identified by numbers at the left of each figure.

Figure 6. Circular permutation analysis demonstrates that ER–associated proteins influence ER–DNA complex formation and distortion of ERE–containing DNA fragments. BERE–, ESeph–, and EATP–purified proteins were incubated with 427 basepair ^{32}P–labeled DNA fragments that had been isolated from the circular permutation vector EREBend I [26] after digestion with Eco RI, Hind III, Eco RV, Nhe I, or Bam HI (RI, H, RV, N, and B) and end labeling with [γ^{32}P]ATP. The protein–DNA mixtures were fractionated on an 8% nondenaturing polyacrylamide gel. The gel was dried and radioactive bands were visualized by autoradiography. ER–DNA complexes are identified by numbers at the left of the figure.

Figure 7. Phasing analysis demonstrates that BERE–, ESeph–, and EATP–purified proteins induce directed bends in ER–containing DNA fragments. BERE–, ESeph–, and EATP–purified proteins were incubated with 281–291 basepairs ^{32}P–labeled DNA fragments containing an intrinsic DNA bend separated from a consensus ERE by 26, 28, 30, 32, 34, or 36 basepairs. ER–DNA mixtures were factionated on a nondenaturing acrylamide gel. The gel was dried and subjected to autoradiography. ER–DNA complexes are identified by numbers at the left of the figure.

Figure 8. Model of ER-associated proteins. This model is based on our current results as well as some additional data reported by others for ER. The model depicts the hormone dependent dissociation of hsp90, as well as significant conformational changes associated with hormone binding, dimerization, and DNA binding. Further dissociation of ER-associated proteins can occur in the presence of estradiol, namely loss of hsp70. However, in the presence of an ERE, both PDI and hsp70 are retained. Significantly, the presence of p45 and p48 appear to be necessary for high capacity ER-ERE interaction. The contact sites between ER and p45, p48, PDI and hsp70 are unknown, as are the true stoichiometric relationships among the proteins present in the complexes depicted.
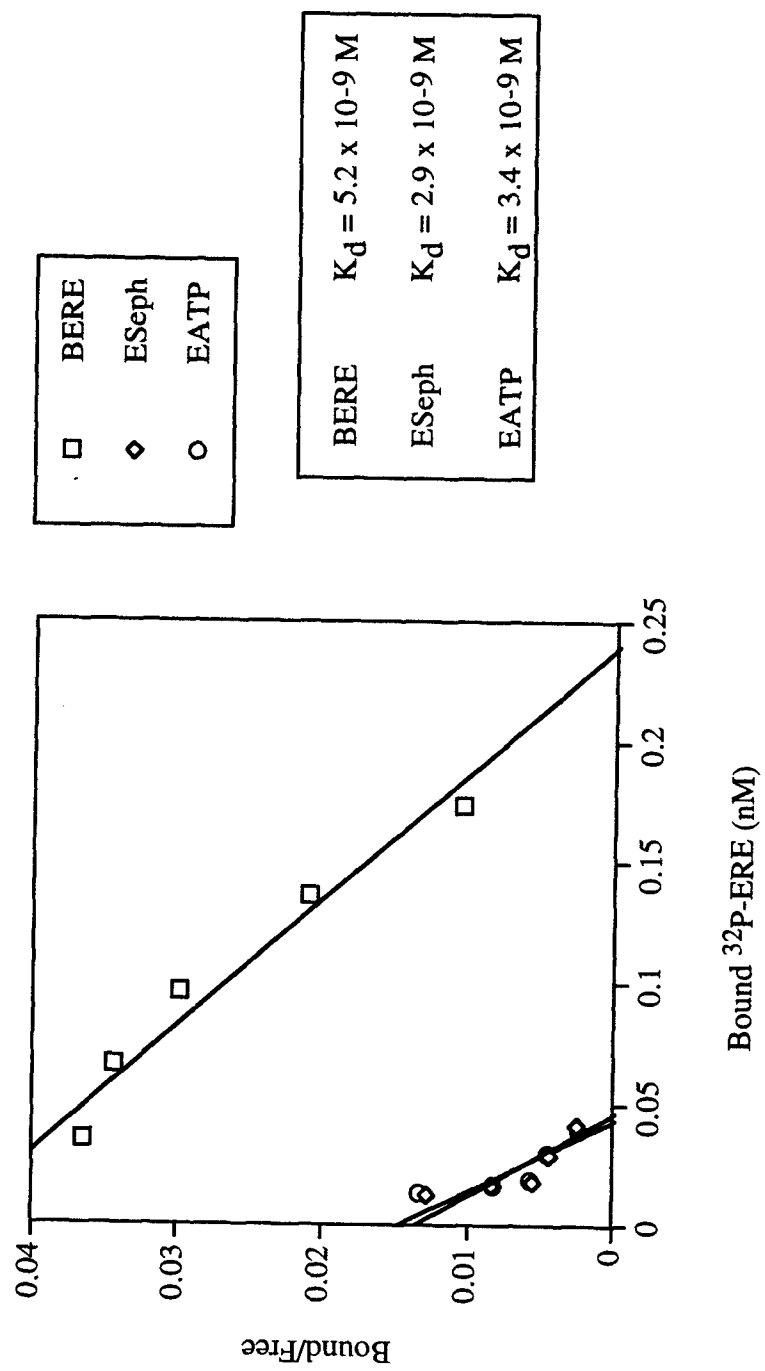
Figure 1

ERE Competitor

Relative ^{32}P-ERE Binding

PRE Competitor

Relative ^{32}P-ERE Binding

No added competitor

Relative ^{32}P-ERE Binding

mtERE Competitor

Relative ^{32}P-ERE Binding

Time (min)

NE
BERE
ESeph
EATP

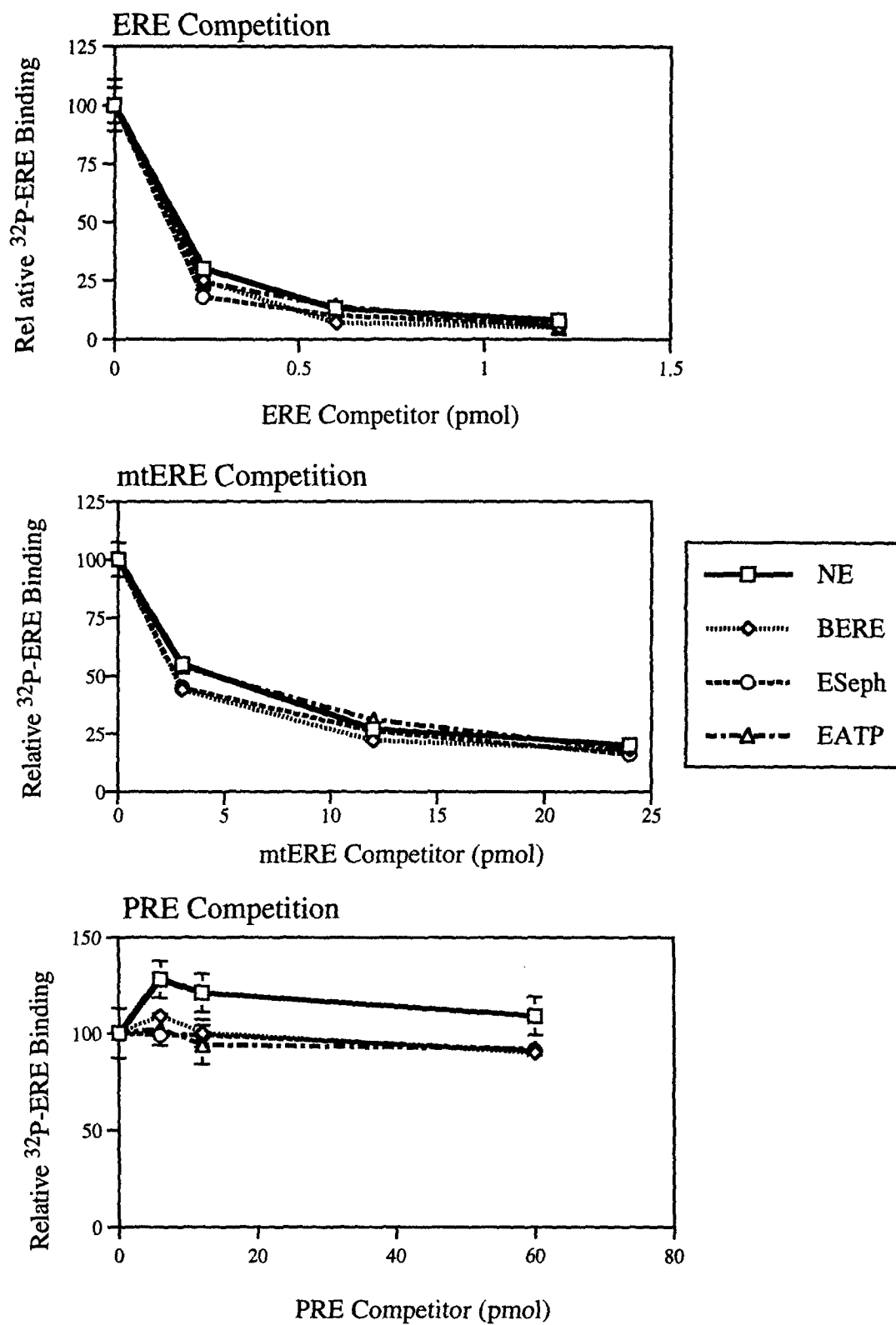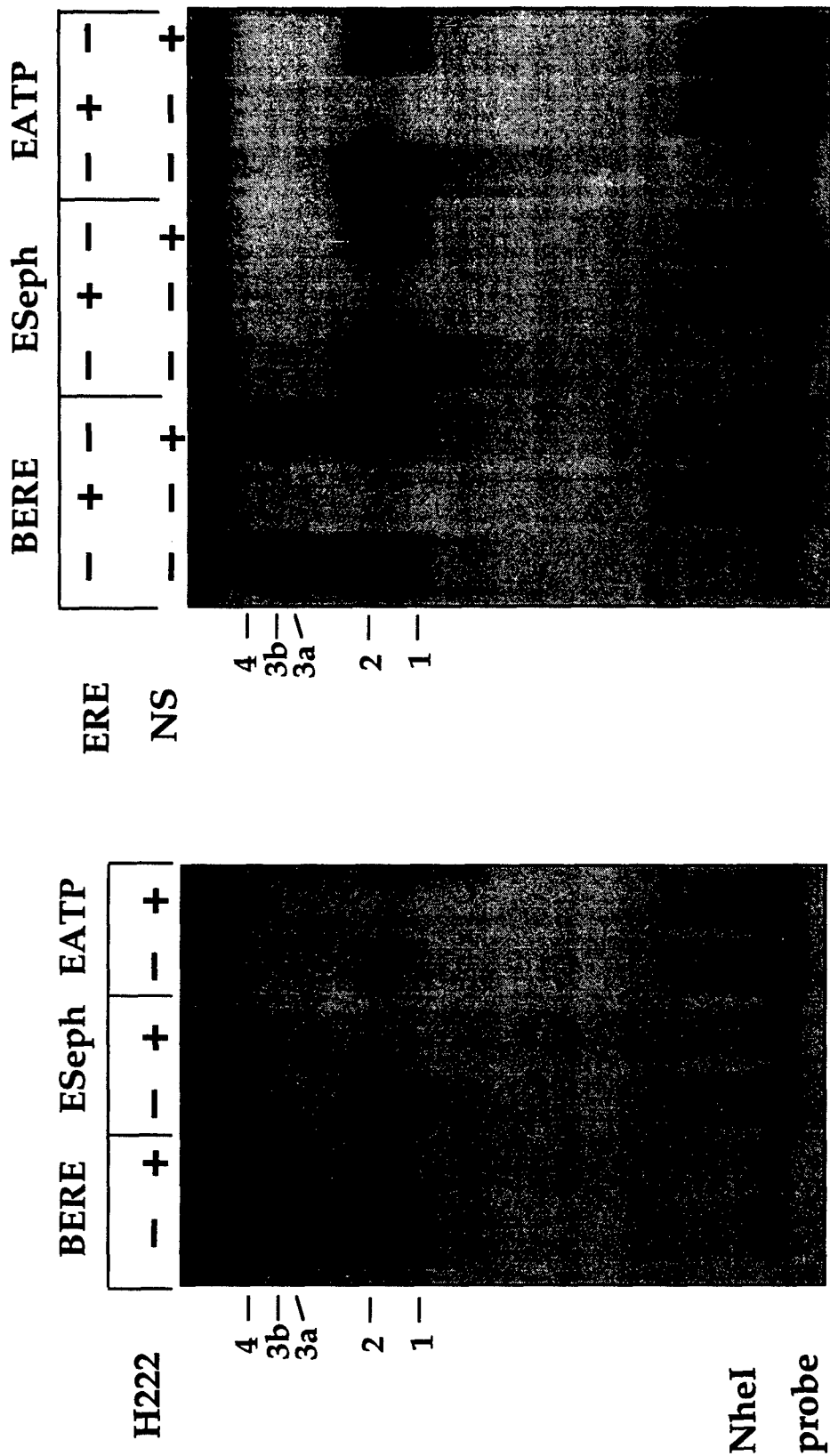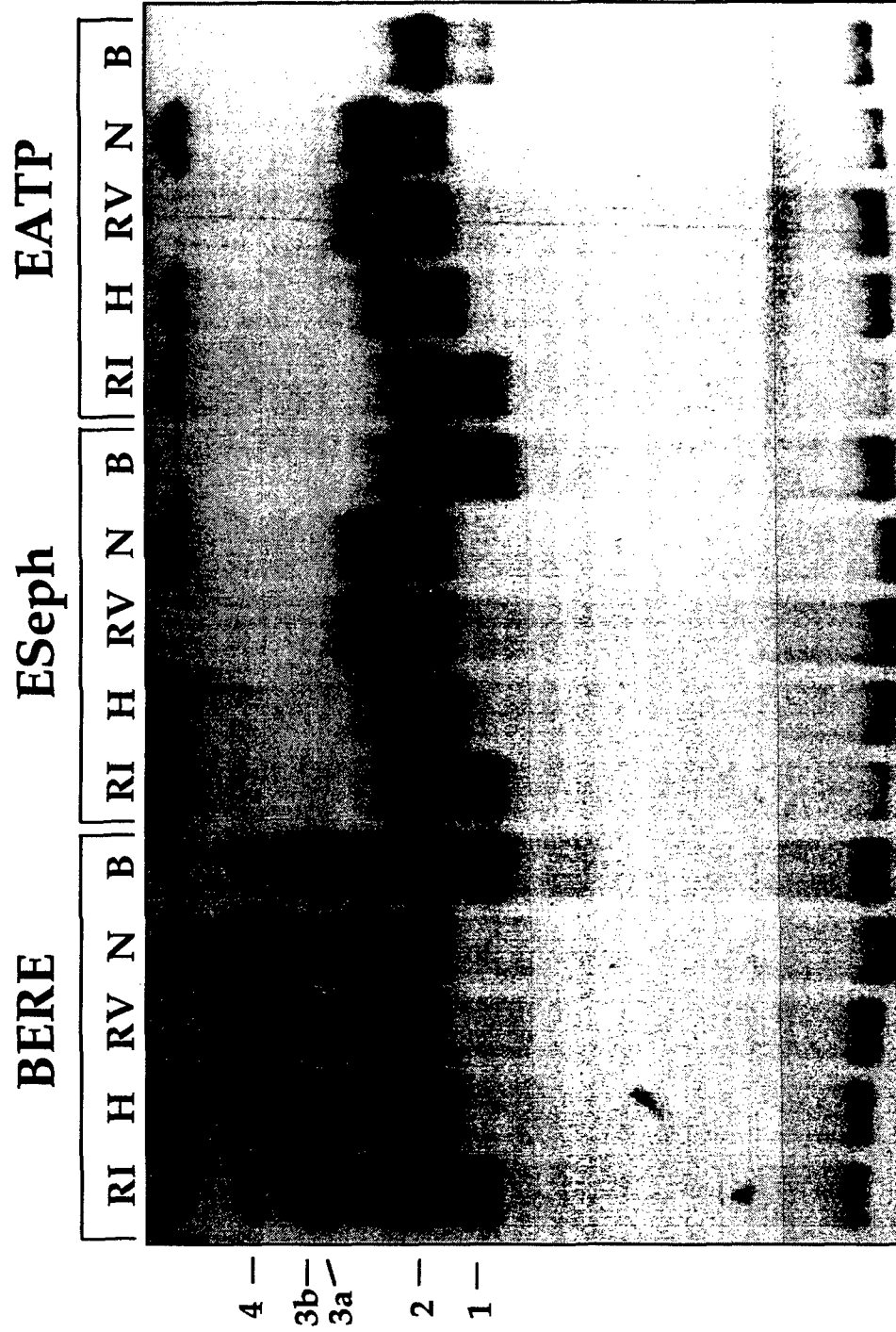Figure 2

Figure 3

ERE Competition

mtERE Competition

PRE Competition
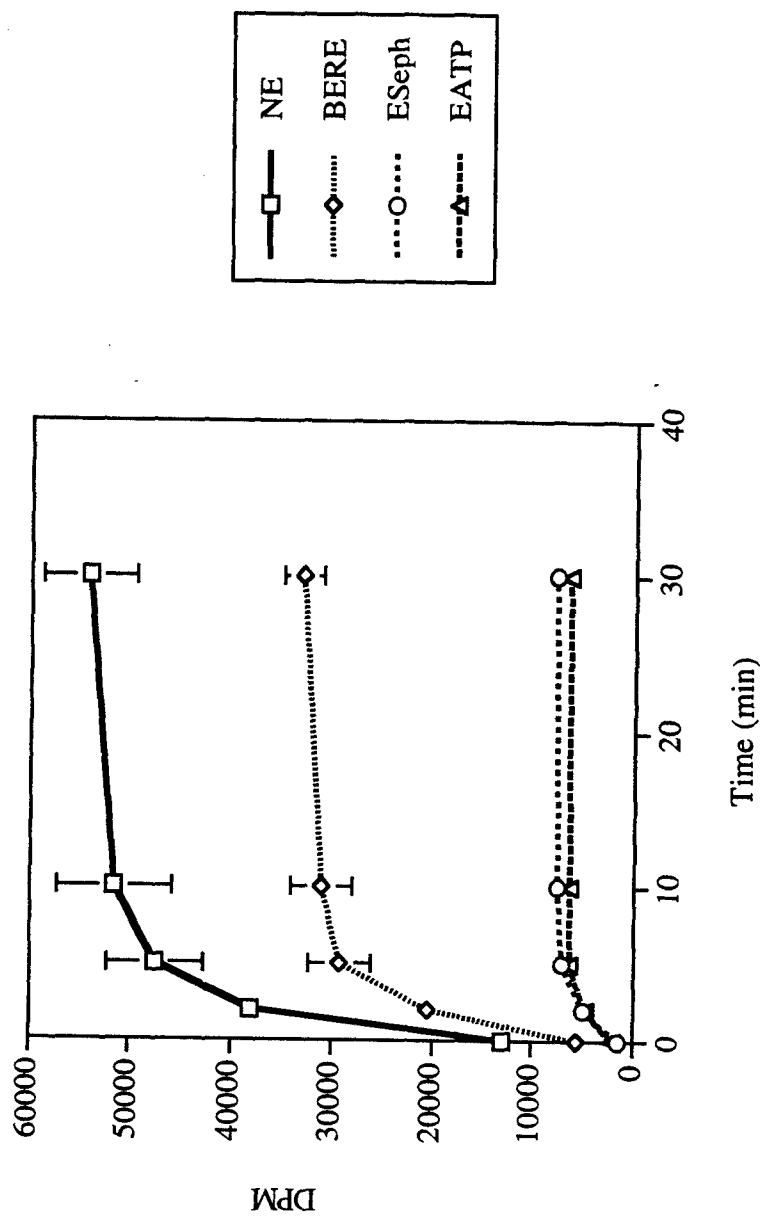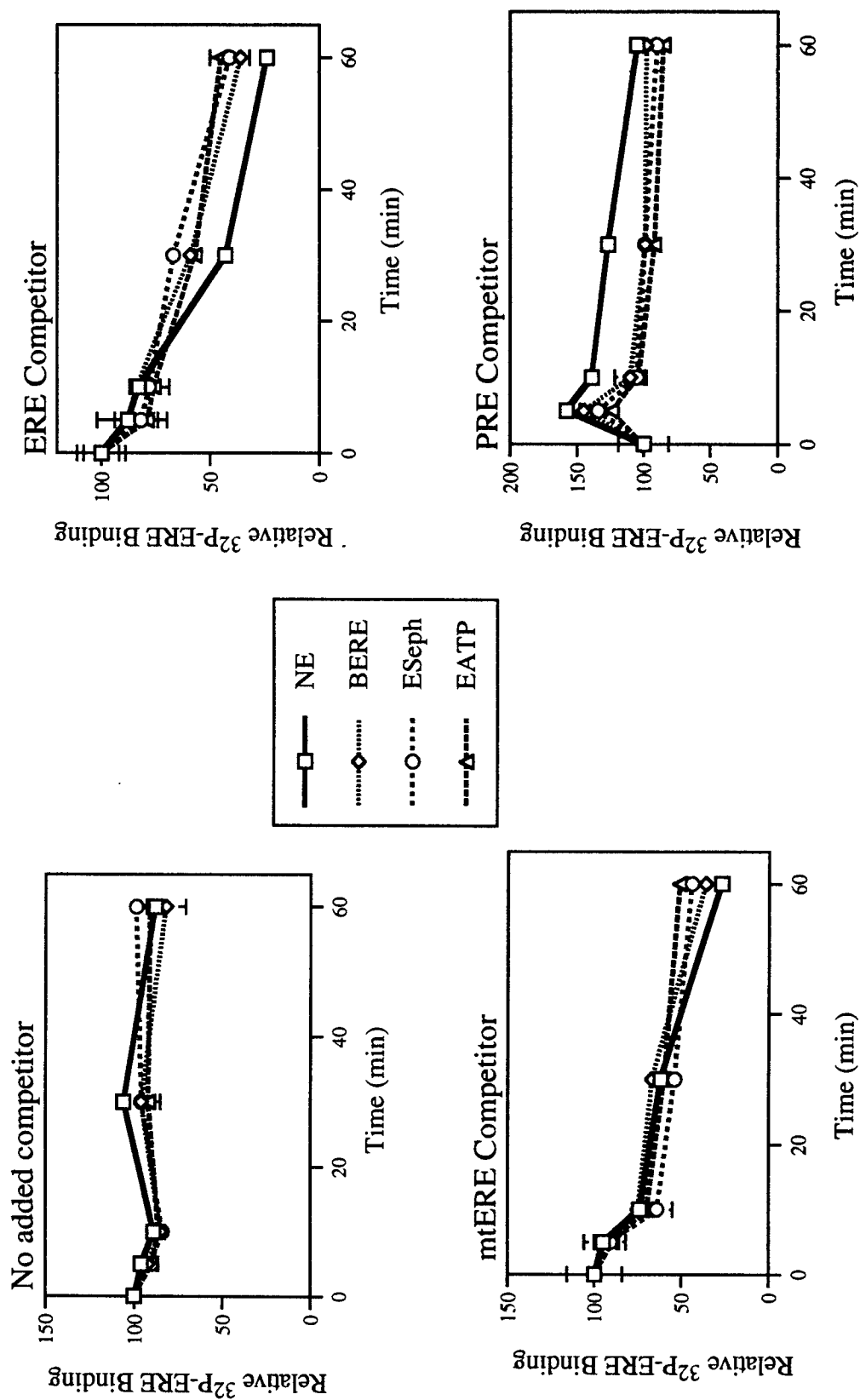
Figure 4

Figure 5

Figure 6

Figure 7

Figure 8